

Editorial

IET Synthetic Biology

To date, biological research has focused on the analysis of naturally evolved systems. These living systems are characterised by complexity, non-linearity and parallelism, often involving multicellular organisms with tens of thousands of genetically encoded components and possessing feedback-dominated mechanisms for self-organisation, reproduction and repair. They produce functional structures that are many orders of magnitude more complex than the most sophisticated man-made artefacts.

A formidable array of biochemical, biophysical and genetic techniques have been assembled for the description of biological systems, and this has given us methods for the comprehensive description of an organism's genome, gene expression patterns and metabolic activities. New imaging techniques allow non-invasive monitoring of biological activities and precise reconstruction of cellular and tissue architecture.

However, there is a general acceptance that the behaviour of complex biological systems cannot be derived purely from the properties of its component parts, and that description of the dynamic interactions within a system is required to encapsulate the behaviour of an entire system. This conception and the availability of large scale datasets from various 'omics' approaches has spawned the new field of systems biology, where computational tools are being harnessed for the numerical description and encapsulation of entire systems. This field is bringing together biologists, mathematicians and computer scientists, and providing a fertile source of new tools and approaches for describing and modelling biological systems. In addition, these developments provide tools for the design and modelling of novel artificial systems.

Recombinant DNA techniques were established in the early 1970s and have become increasingly capable since. Further, DNA synthesis techniques have become cheaper and ever-larger-scale assemblies of artificial genetic material are becoming feasible. For some years, scientists such as Tom Knight at MIT have realised that genes might be fashioned as modular building blocks for general-purpose use as genetic logic elements, and that the impending collision between new technologies for design and assembly of genetic systems will provide opportunities for a fundamental rethink in our approach to biology and its application.

Synthetic biology is the discipline that has resulted from this collision of new enabling technologies. Thus, recombinant DNA and improved DNA synthesis techniques provide the means of assembling new genetic systems, and computational approaches borrowed from systems biology provide tools for the design and modelling of artificial biological circuits. In addition however, the shift from analysis of naturally evolved biological systems to the construction of synthetic systems requires the recruitment of engineering principles to biology.

In principle, simple biological elements can be adopted as reusable components, which are well characterised and can

be used for the construction of more complex devices and systems. This approach requires the application of engineering concepts such as modularity, reusability, abstraction and insulation from underlying detail. The reuse of modular components also facilitates software modelling, and work in the field is promoting parallel developments in computer software. New students and workers are coming into the field from very diverse areas, and need to come to grips with the nitty-gritty of unfamiliar biological systems, engineering tools and computer sciences. There is a growing demand for specialised coverage of this new field, including educational and review materials.

The Institution of Engineering and Technology (IET) was formed in 2006 after the merging of the Institution of Electrical Engineers (IEE) and the Institution of Incorporated Engineers (IIE) and now has more than 150,000 members worldwide. It is the largest professional engineering society in Europe and the second largest of its type in the world. The IET is a non-profit organisation that provides a wide range of services and information for technical research and education, including publication of a number of research journals. The IET has launched this new journal, *IET Synthetic Biology*, with the aim of supporting this growing new community. The journal will publish conventional research papers in synthetic biology in addition to providing a 'nuts and bolts' view of this new field, as well as review and educational materials. In particular, we wish to support the activities of young workers entering the synthetic biology field. Publication in *IET Synthetic Biology* will be free of page charges.

Synthetic biology is a very young and interdisciplinary field. In addition to conventional research and review articles, we see an important need for practical articles describing technical advances and innovative methods useful in synthetic biology. We will encourage submission of technical articles that might describe novel BioBrick components, construction techniques, characterisation of a new biological circuit, new software or a practical 'hands-on' guide to the construction of new instrumentation or a biological device. Further, we will provide a short report format which we hope will provide an easier avenue for publication by talented undergraduates in the field. We believe that this is needed in a field where the international Genetically Engineered Machine (iGEM) competition plays such a crucial role in recruiting and energising undergraduate students and faculty alike. In addition to the print journal, we are developing associated web resources. These will include a repository of online video resources, specialised review material and research tools for synthetic biology. For the early stages of the journal's development, published content will be freely available online as PDFs.

At this early stage of development in the field, we aim to publish thematic issues, with topical review content. We hope that central themes will provide a focus for new readers. This first issue is an example of this, where we

are focusing on the activities of the last iGEM competition, the BioBrick repository and their impact on the field of synthetic biology. The iGEM competition has played a special role in the development of synthetic biology as a field. It has provided a platform for advocacy of the underlying engineering principles, for the development of an open repository of modular BioBricks and their worldwide distribution, and not least, the recruitment of many enthusiastic students directly into the field. It has provided the outline of new paradigm for biological teaching. Undergraduate teaching in biology has shifted away from a practical approach to cramming, as our knowledge of natural systems increases. In contrast, there is generally a much stronger emphasis on training in problem-solving skills for undergraduate engineering students. The iGEM competition mixes student engineers and biologists, and provides them with exciting challenges in scientific design, laboratory practice and project management, which are largely outside the normal undergraduate experience, and at the cutting edge of a new field. This issue contains an overview of the iGEM competition from James Brown, an iGEM 2005 team participant, iGEM 2006 student ambassador and iGEM 2007 team advisor. The issue also contains team reports from a wide variety of teams that participated in the last iGEM competition.

We hope that this year's teams will also be encouraged to publish their team's efforts, and that this special *IET Synthetic Biology* iGEM issue will become an annual event.

For some of us, the growing application of engineering principles to biological design and construction marks a practical transition for biological research. For example, work in the physical sciences has been transformed completely over the last half century by the impact of human engineered constructs. Basic research on electronic phenomena and solid-state physics has given rise to new fields and entire industries devoted to microelectronics, optics and software development which dwarf their origins. One might expect a similar shift in biological research as synthetic biology begins to offer improved rational design and reprogramming of biological systems. If so, synthetic biology will contribute to future improvements in the microbial, plant and animal cell engineering that are clearly needed for the renewable technologies of the 21st century.

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Editor-in-Chief

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The iGEM competition: building with biology

J. Brown

Abstract: Can simple biological systems be built from standard, interchangeable parts and operated in living cells? Or is biology simply too complicated to be engineered in this way? The international Genetically Engineered Machine Competition (iGEM) is an open design challenge for student teams that addresses this difficult question. Using a library of standardised parts known as BioBricks, groups of undergraduates from around the world spend their summer designing and assembling biological devices, to build genetic machines.

1 Introduction

The engineering of new biological systems is an exciting frontier, with opportunities for collaboration between biologists, programmers and engineers. The iGEM competition throws together students from different disciplines, requires them to initiate a novel scientific programme over the summer, and challenges them to learn and share different skills. The competition has provided a new educational model in an exciting new field. In Cambridge, we are unreservedly positive about the educational aspect of the competition. As well as learning challenging new scientific skills, the competition allows students to experience project brainstorming, management, teamwork, presentation and other organisational skills in a way that is essentially outside the undergraduate curriculum. The competition provides a powerful educational tool, exposing students to engineering challenges and a modern research environment, while in pursuit of their own goals.

The iGEM competition's long-term goals are to enable the systematic engineering of biology, while promoting the open and transparent development of relevant tools. To this end, founders Tom Knight and Randy Rettberg of MIT's Computer Science and Artificial Intelligence Laboratory, and Drew Endy from the Biological Engineering Division of MIT, have pioneered the collection and use of a library of modular biological components. They have established the Registry of Standard Biological Parts (<http://parts.mit.edu>), which includes several hundred basic parts such as operators, protein coding regions and transcriptional terminators. It also includes many devices such as logic gates and input/output modules built from these basic parts. These parts and devices are known as BioBricks [1].

2 The competition's history

Knight, Endy and Rettberg took their first steps towards realising an international competition in synthetic biology back

in 2003 with the help of fellow computer scientist Gerry Sussman. The four established a class, providing a 'hands-on introduction to the design and fabrication of synthetic biological systems' for MIT's IAP (Independent Activities Period), a four week session put aside in MIT's calendar for undergraduates to engage in a wide range of innovative projects that combines learning and fun. A group of 16 students were challenged to improve on Elowitz's Repressilator [2] and construct biological oscillators. The programme proved a success, running again the following year with Pam Silver from Harvard joining the instructors. In IAP 2004, students turned their attention to cellular patterning in bacteria, with final designs ranging from bull's-eyes to polka dots. Both IAP programmes received a substantial DNA synthesis budget and many of the parts are included in the Registry's current distribution.

The IAPs laid the foundations for Rettberg, Knight and Endy to expand the program. The summer of 2004 saw the first US intercollegiate design competition in synthetic biology. Student teams from Boston University, Caltech, MIT, Princeton and UT Austin looked to engineer cellular state machines and counters for SBC (Synthetic Biology Competition) 2004. The competition culminated in the first November Jamboree at MIT, where participants shared their work, experiences and hopes for the future. Campbell's absorbing account of the 2004 Jamboree includes full project descriptions and provides a unique insight into the forerunner of the iGEM competition [3].

The following summer, the competition included international teams for the first time. Student representatives from 13 universities participated in iGEM 2005. North American teams from Berkeley, Caltech, Davidson, Harvard, MIT, Oklahoma, Penn State, Princeton, Toronto, UCSF and UT Austin were joined by European entries from Cambridge and ETH Zurich. Unlike previous competitions, the teams were not given a specific task, but were simply challenged to 'design and test a simple biological system from standard, interchangeable parts and operate it in living cells'. As a result, student projects covered a wide range of subjects. Designs included regulation of chemotaxis, cell-cell communications, biological sketch pads, a digital counter, thermometers, cellular relay races, biological wires and many more. The first weekend of November 2005 saw

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over 150 students and instructors coming together for the Jamboree held at MIT's Stata Center, to share and celebrate their work [4].

3 The ambassador programme

The iGEM competition tripled in size from 13 to 37 teams in 2006, spread throughout North America and Europe, with new entries from South America, India and Japan. With rapid growth came the need for increased team support and programme development. This was addressed through the launch of an ambassador programme. iGEM Director Randy Rettberg approached previous participants of the competition to take lead roles in promoting team–team communication, improving the programme and to ultimately make the teams, projects and competition more successful. Andrew Hessel, Melissa Li and I covered the 22 North American schools. Reshma Shetty looked after the Asian teams while taking a graduate advisory role within the MIT project and Meagan Lizarazo visited the Latin American teams, when not developing the Registry's robotic BioBrick assembly line. Jonas Nart, Tamara Ulrich and Robin Kunzler supported the European teams, based out of ETH Zurich.

An alternating schedule of team visits and programme development back at MIT's Registry proved a useful, if challenging combination. My visits were stimulating and presented an ideal opportunity to not only inform teams about new features and developments, but to get first-hand feedback from students and faculty on the programme, any problems they were facing and many new ideas. During my visits I witnessed a wide range of projects, approaches and problems. It also presented an opportunity to see first hand some of the best researchers in the field interact with their students. The chance to regularly cross paths with Melissa and Andrew at the Registry during gaps in their schedules provided a sound platform to improve the competition format and structure as well as inform development of the Registry. We later addressed programme publicity, the Jamboree and help lay the foundations for iGEM 2007. The ambassador programme proved a unique, challenging and massively rewarding experience that hopefully aided both rookie and experienced iGEM teams alike.

iGEM 2006 proved a huge success. A wide range of projects were presented at the annual Jamboree, many achieving their initial goals. Significantly more designs were realised than in earlier competitions and several hundred new BioBricks were contributed to the Registry. The teams' work is described in detail in this first issue of IET Synthetic Biology.

4 BioBricks

BioBricks are standard interchangeable parts, developed with a view to building biological systems in living cells. BioBrick parts can be assembled to form useful devices, through a process referred to as 'standard assembly'. This uses normal recombinant DNA manipulation techniques based on restriction enzymes, purification, ligation and transformation. For example, two BioBrick parts, B0034 (blue) and C0010 (green), can be assembled to form a composite B0034–C0010 (blue–green) part via standard assembly, illustrated in Fig. 1. Every BioBrick is flanked by restriction sites, comprising the BioBrick prefix and suffix, including EcoRI and XbaI cut sites on the left (prefix) and SpeI and PstI on the right (suffix).

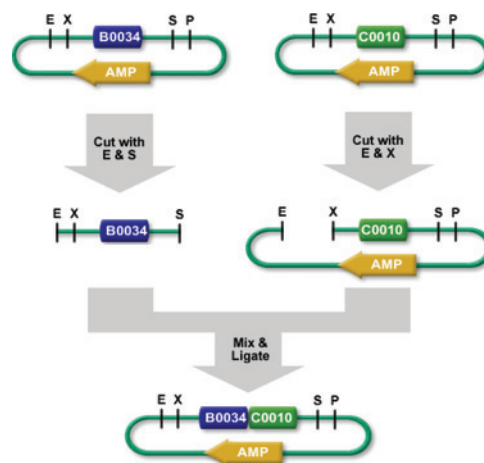


Fig. 1 Standard assembly example

Using normal recombinant DNA manipulation techniques based on restriction enzymes, purification, ligation and transformation, two BioBrick parts, B0034 (blue) and C0010 (green), can be assembled to form a composite B0034–C0010 (blue–green) part via standard assembly

For the assembly shown, the part B0034 (blue) is cut out of its plasmid with the enzymes EcoRI and SpeI. The result is called the insert because it will be inserted into the plasmid containing the other part. In a separate reaction, a hole is cut in the plasmid containing the part C0010 (green) using EcoRI and XbaI. Using gel electrophoresis, the insert for B0010 (blue) and the cut plasmid containing C0010 (green) are purified and the unwanted fragments discarded. The purified insert and cut plasmid are mixed under the right conditions to allow ligation of the two EcoRI sticky ends and joining of the compatible SpeI and XbaI sticky ends, which destroys the internal cut sites. The DNA backbone contains a composite B0010–C0010 (blue–green) part and can be transformed into competent *E. coli* cells. These cells may be grown to produce as much of the new BioBrick (B0010–C0010) as desired. An alternative to BioBrick standard assembly is three antibiotic (3A) assembly. A description can be found in the Registry's help section at parts.mit.edu.

Using standardised parts has many advantages. It isolates the construction procedure from the design process, provides a repeatable method allowing BioBricks to be made longer and more complex in function while the construction process remains identical, confers full compatibility to all the BioBricks in the collection, and significantly, means each independent project which uses BioBricks serves to add a larger range of parts and devices to the ever-increasing range of modular components in the Registry of Standard Biological Parts.

5 The Registry of Standard Biological Parts

Information on each BioBrick is stored in the Registry's online library at <http://parts.mit.edu>. BioBrick parts, devices and systems are arranged in the Registry within an abstraction hierarchy, as proposed in Endy's Foundations for Engineering Biology [5]. Each online record is entirely customisable, allowing the display of relevant information about the part. There are five subsections to each online BioBrick entry (Fig. 2):

1. The 'Main Page' gives an overview of the part and can provide links to appropriate data or specific subpages.

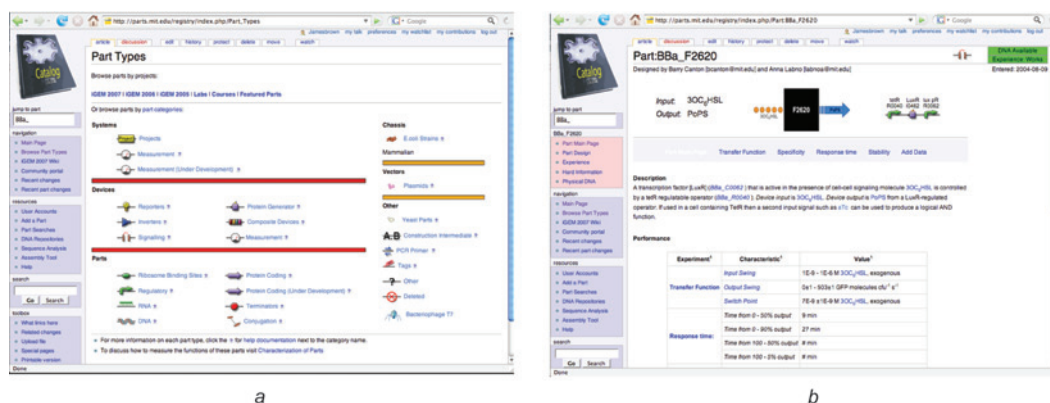


Fig. 2 Screenshots from MIT's Registry of Standard Biological Parts at <http://parts.mit.edu>

a The 'Part Types' page from showing BioBricks within an abstraction hierarchy

b A good example of a well-characterised part (BBa_F2620 by Barry Canton and Anna Labno)

2. The 'Part Design' section details design considerations, where the part was sourced and any relevant references.
3. The 'Experience' section allows other users to rate and leave comments on the BioBrick, indicating a reliable working part or highlighting any issues, much like an online book or DVD review.
4. The 'Hard Information' page simply contains the data that allow the Registry to run efficiently as a searchable database.
5. The 'Physical DNA' section includes an exhaustive list of all versions of that BioBrick, which plasmid it resides in and from which well an iGEM participant can obtain the DNA, from the current distribution of four 384 well source plates.

The Registry provides several tools that aid the design and understanding of BioBrick systems and devices. Users can easily 'Add a Part' to the Registry with a few simple clicks, pasting in sequence information and annotation. The 'Part Searches' tool allows users to quickly and easily scour the Registry for information, trace the origin of more complex parts and list associated sub-assemblies, and see which higher systems have been constructed with any part of choice. The 'Sequence Analysis' tool is used to organise and analyse a set of DNA sequencing runs by comparing DNA sequences against parts in the Registry. The 'BioBrick Repository' maintains information on the DNA of the BioBrick parts in plasmids and cells. The same functional part such as a particular Quad Part Inverter may be available in different cell strains or plasmids and may have been built with different assembly techniques resulting in different scars between its components. All of this information is stored in the repository database. Finally, the 'Users & Groups' tool allows users to manage their user information, see their groups, join new groups, and find names to use for new parts here.

6 What lies ahead?

The iGEM competition has proved a valuable educational tool, inspiring many young minds from a multitude of disciplines to pursue further study in synthetic biology. It is uniquely positioned to place students at the cutting edge of an exciting young research field that holds huge potential. As the competition has grown rapidly over the previous years, correspondingly so has the collection and complexity of available BioBricks. This growth looks set to continue, both in terms of iGEM teams, geographical spread and

available BioBricks. Significantly, established laboratories, many of them advocates of iGEM, have started to contribute to the BioBrick collection through their day-to-day research and in doing so look set to provide a range of better characterised parts and devices.

The Registry's biggest shortcoming at this early stage in its development is the varying quality of its BioBricks. The iGEM competition's short ten-week summer period means a large proportion of the parts are not well documented and contain inaccurate information. There are currently very few well-characterised, accurate parts available. The Registry's founders are very much aware of this issue and are actively taking steps to address it. A system is being put in place that would see official BioBrick status given to parts that meet established quality criteria and pass peer review. Tom Knight is to chair the review committee and he is looking for members who will first decide how the process should work and then organise the reviews. If you are interested, please state your interest online on the Registry's website.

The future promises many exciting developments. The introduction of new software and standards to aid the design and modelling of increasingly complex engineered biological systems. The falling cost of DNA synthesis looks set to eliminate the laborious, difficult, time-consuming process of construction, replacing standard assembly with an online order form for designed BioBrick systems. Free from the constraints of traditional DNA manipulation techniques and equipped with these new software tools, this new era of biotechnology promises more complex and effective biological devices, aiding research and providing end applications alike. There is no doubt in my mind of the growing importance of synthetic biology and its potential to address prominent global issues relating to energy, the environment and healthcare. Synthetic biology looks set to fuel a new generation of biology-based industries and certainly has a very bright future. So too does the iGEM competition, which has contributed so much to the growth and prosperity of synthetic biology over the last four years. We can expect many past and future iGEM participants to find themselves working for or even running this new generation of bio-based industries.

iGEM 2007 is underway with almost 60 teams participating from Asia, North America, Central and South America, Australia, the UK and Europe. Ahead of all of them lies an exciting summer of hard work mixed with enjoyment, as they strive to design and build novel biological systems. We can all look forward to an exciting Jamboree in

November and another vintage of inspired synthetic biologists.

7 Acknowledgments

I'd like to thank Randy Rettberg, Drew Endy and Tom Knight for the opportunity to take on the role of iGEM ambassador and having me to stay at the Registry last summer, as well as the other ambassadors and all of the participants in IAP 03/04, SBC 2004, iGEM 2005 and 2006 who have contributed so much to the competition's development and growth.

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Barbie nanoatelier

A. Kuznetsov

Abstract: We use a DNA origami technique to design a strand of DNA that wraps into a meaningful shape. First, the DNA folds into a two-dimensional rectangular sheet, or DNA platform. Second, this sheet turns into a short pipe. Next, these pipes form long pipes or tetrahedrons, which will assemble into more sophisticated structures. Once the process of DNA folding into 3D objects is understood, shapes can be chosen arbitrarily. We propose a database for DNA parts (e.g. aptamers) that could be inserted into staples to form a desirable pattern and to anchor specific molecules. The origami technique is considered to be suitable for molecular logic and/or processing.

1 Introduction

'The best way to predict the future is to invent it.'
Alan Kay, Xerox PARC, 1971

When Francis Crick was too lazy to explain everybody that his job was a mixture of physics, crystallography and biochemistry, he invented the term 'molecular biology'. Ironically, I wrote 'synthetic biology' in my CV to express my broad interests in molecular biology and genetics, structural and mathematical biology, developmental biology, and evolution. Christopher Nehaniv used the term 'constructive biology' in an attempt to understand natural life using an engineering approach. It was a remarkable way of thinking. Richard Feynman famously wrote: 'What I cannot create I do not understand'. My understanding is that 'constructive biology' means: (1) a collection of building primitives; (2) their standardisation and abstraction; and (3) the development of design rules that were briefly demonstrated by Stratagene, Promega, Invitrogen or Eurogenetics in the biotechnology industry many years ago and were emphasised recently in academia [1, 2]. Constructive biology [3] includes genetic engineering, cell and tissue engineering, and leads to a hierarchical design in biology that occurs naturally [4]. The neat term 'artificial life' has been used by the scientific community after Chris Langton organised the first International Conference on the Synthesis and Simulation of Living Systems at the Los Alamos National Laboratory in 1987. I cannot exclude the possibility to extend the 'artificial life' approach into physics, chemistry or biology to exploit mediums beyond that of silicon. It is very reasonable to search for alternative forms of computation, such as quantum and DNA computing. The term 'synthetic biology' is more exciting in my opinion, because it will need a set of completely synthetic building blocks to create synthetic (orthogonal) life. If we can synthesise DNA, predict perfectly protein folding, calculate protein–protein and protein–DNA interactions,

why can't we design a whole organism from scratch and say proudly – 'synthetic biology'?

2 Synthetic biology in Freiburg

The fields of biocomputing and synthetic biology have been familiar concepts at Freiburg University since 2003. Klaus Palme was perhaps the first to realise a future role for synthetic biology. The related discipline of systems biology is promoted by Jens Timmer and Ralf Baumeister. I was stimulated by Stephen Wolfram [5] in general and particularly by Leonard Adleman [6] and Paul Rothmund [7] to mimic natural computational processes [8] as well as to popularise the ideas of unconventional computation and engineering biology, following the work of Tom Knight [9] and Drew Endy [1]. Hubert Bernauer studied this emerging science to develop policy recommendations for market. An active participation in the Synbiology NEST project [10] gave us opportunities to take a common picture of this new field, comparing it to nanotechnology and artificial life science. We realised there was a great need for a special education program in synthetic biology.

To achieve effective communication, cooperation, and social self-organisation we have established the Gene Engineered Machines Freiburg Club, later christened the 'Barbie Nanoatelier'. This was attended by all the students of the self-education program [11]. We educate students in four different areas:

- (1) biological background—green line
- (2) programming and simulation—red line
- (3) synthetic chemistry/biology tools—magenta line
- (4) system approach—blue line [12]

The first part of this program was used by Kristian Mueller, Katja Arndt, and Albrecht Sippel to organise a seminar entitled 'From Gene To Machine'. We started with a brainstorming session and preliminary training in spring 2006, followed by intensive research during the summer. Faculty, post-docs, and graduate students from applied science, biological, chemical, and physical departments were highly encouraged to participate as advisers to the underground team. Finally we had a student team of five members: Daniel Hautzinger (biologist, Masters student), Olga Soboleva (engineer, undergraduate), Irina Petrova (biologist, post-graduate student), Yutthaphong

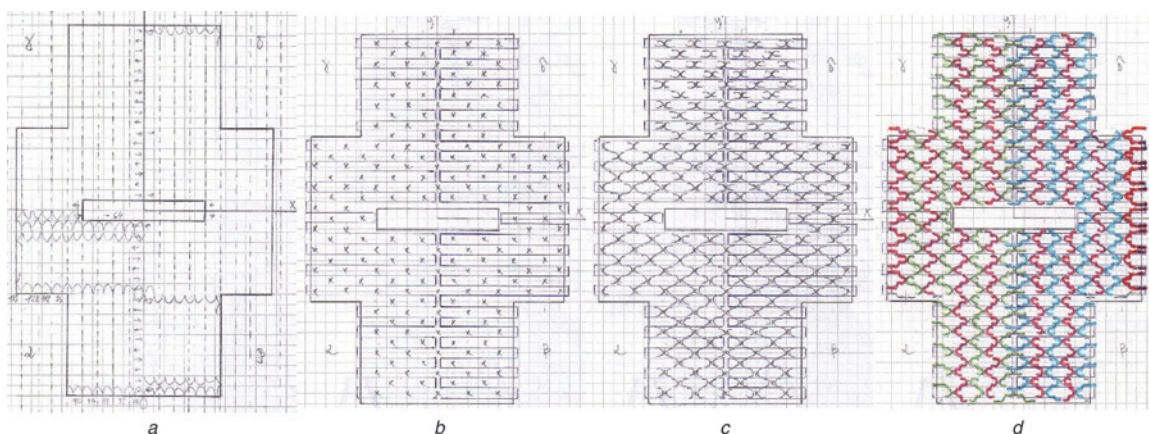


Fig. 1 *T-shirt (planar representation)*

- a* Fill the shape with building blocks
- b* Draw the raster with a single long scaffold strand
- c* Add helper strands to bind the scaffold together
- d* Merge helper strands to enhance the scaffold

Phongbunchoo (biologist, Masters student), and Mona Klein (engineer, graduate). Prof. Jan Korvink held the chair of the team, Dr Andrew Kuznetsov was responsible for the education, and Dr Svetlana Santer for atom force microscopy (AFM).

3 DNA folding project

Even on a level of basic components, biology can be complex and unpredictable. It makes sense to keep things as simple as possible. Open discussions allowed us to choose a ‘hot’ scientific topic. Recently, a new approach based on DNA complementarity has been proposed by Paul Rothemund to create complex 2D structures made of DNA fragments [13]. This method, referred to as the DNA raster fill method (scaffolded origami), can be used to create nanometer-scale planar structures of arbitrary shape and to attach an addressed molecular pattern with 6 nm scale resolution onto the knitted DNA surface. The goal of the project was to use this new technique in order to: (1) train students on DNA nanotechnology and synthetic biology; and (2) highlight a new research perspective.

During the project, we taught students not only to repeat previous experiments, but also to generate new ideas and scientific goals. For instance, the technique is extended to 3D self-assembled DNA sheet structures. T-shirt-like objects that consist of two intersected cylinders ($\varnothing \sim 15\text{--}20\text{ nm}$) with a logo on the front side and a hole for a ‘neck’ were

designed by the team (Figs. 1 and 2). The students used M13mp18 DNA as a scaffold and a set of oligonucleotides as staples to fold the viral DNA into the particular shape to analyse it under AFM and by spectroscopy.

Firstly, all students were encouraged to study Rothemund’s original paper, supplementary materials and some additional articles and reviews related to DNA nanotechnology (e.g. [14–17]). Students found that 1D tracks, 2D grids, and 3D scaffolds of DNA have been used successfully as nanotemplates for programmable self-assembly. They realised that Rothemund’s method is an easy and reliable approach to create sheets from DNA with a desirable pattern.

Next, students were asked to design some 3D structures using the DNA sheets. They made sketches of carpets, strips, tubes, and even a Nike® ‘blouse’ for an imaginary Barbie doll about 100 nm tall (Fig. 2). The team designed oligonucleotides for a rectangular DNA sheet, an IMTEK logo, tubes, tetrahedron and a T-shirt.

After that, we studied new methods of parallel throughput synthesis of DNA on chips and introduced students into the work in a wet lab [18]. Students studied the theory of AFM (tapping mode) to visualise intermediate plane DNA structures as well as the analysis of 3D DNA objects using optic methods (fluorescence shift, correlation spectroscopy). Quantum dots (QDs) were considered for the trap analysis and oligonucleotides labelled with fluorescence markers were suggested to investigate the desirable junctions of specific edges. Students also learned methods of single molecule detection.



Fig. 2 *Front part of Nike® blouse*

- a* Rectilinear merge pattern
- b* Staggered merge pattern

Finally, students learned to collect, analyse and present data on a wiki. The obtained results and experiences which will be used by students in the future for diploma and PhD theses [19].

4 Results

Our team achieved some unexpected results during the iGEM2006 competition [20]:

- we started a new branch in the BioBricks repository for DNA origami parts, devices and systems
- following Paul Rothmund's work, students created a universal DNA platform for a pattern on the nanometer scale that will allow us to organise chemical reactions, assemble and pattern QDs
- proposed a correlation spectroscopy/microscopy for the investigation of DNA folding
- designed the tetrahedron 'Nautilus', which is a 3D DNA building block for a hierarchical assembly, nanoswarm and amorphous computing
- suggested potential applications in cryptography for DNA origami
- emphasised aesthetic principles of artificial life design
- founded 'Barbie Nanoatelier: Open Source DNA nanotechnology' – the way to artificial life and artificial intelligence.

We dramatically simplified Rothmund's scaffold origami method. Students needed only a browser with access to standard bioinformatic tools and a text processor, if they did not make too complex a design.

4.1 Abstraction instructions

1. Take a block of paper: 1.5 block on paper = 1 building block of 16 nucleotides = 1.5 turn DNA = 5.4 nm horizontal and 4 nm vertical. Put a given transparent shape on the paper to fill the shape by blocks.
2. Find a 'snake' path through the Manhattan geometry horizontally with turns in the vertical direction, trying to exploit symmetry.
3. Starting at one end of the DNA strand, insert a crossover to the strand section above every alternate building block. Add helper strands to bind the scaffold together. As designed first, most staples bind two helices and are 16-mers.
4. Merge helper strands to enhance the scaffold. As designed second, most staples bind three helices and are 32-mers.
5. Fill up the scaffold with letters A, T, G, C, defining corresponding staple sequences by complementary mapping from scaffold to valid sequence (A, T and G, C).
6. We now have one long scaffold and many shorter staples.

4.2 Implementation instructions

1. Send the request to a DNA synthesis company. You will receive two small bottles: one with the scaffold DNA, the other full of staples in 1xTAE (pH 7-8.4) buffer with 10 mM MgAc.
2. Get the following equipment: pipettes, 'cooler' (gradient PCR machine), AFM, mica.
3. Mix the scaffold and staple DNAs in 1/10 (mol/mol) proportion ($2 \times 50 \mu\text{l}$).
4. Heat to 92°C and program the cooling down to room temperature 20°C , over 16 h.
5. Cleave the mica and place $5 \mu\text{l}$ droplet on the mica.
6. Image with AFM.

For the iGEM 2006 competition, we proposed an external BioBrick repository for DNA nanotechnology and added examples of DNA building blocks. Our students used the database space in MIT Registry of Standard Biological Parts [21] from BBa_J35000 to BBa_J35999 to divide it into two major categories: (1) structural DNAs; and (2) protein-coding DNAs. The structural DNAs were organised in a hierarchical manner: structures, devices and systems (Fig. 3).

Examples of a new class of BioBricks (called origami BioBricks) included: GCN4 bZIP binding motive [22], B-Z DNA switcher [23], and arms with different numbers of fingers, which could be a prototype for DNA aptamers and hairpins for a relief patterning (Figs. 4 and 5). The adapted mFold algorithm was used to predict DNA secondary structures [24].

5 Why nanotechnology?

The basis for engineering on the nanometer scale is a library of molecular primitives (finite elements) that could be interchanged in a desirable way for a bottom-up design [25]. The general aim of the project was to discover those primitives. We created a universal addressable platform with 6 nm scale resolution to compose these primitives in a desirable, precise and functional way. The building blocks with different types of interactions should assemble in a hierarchical way. The molecular system assembles in such a way that the resulting objects also self-assemble into the superstructure, and so on. We try to implement a simple computation and organise an interaction/communication between smart nanoparticles to achieve a dynamical self-organisation process with the functional tasks like foraging (i.e. 'swarm nanointelligence' [26]). The computational power of the system will also be estimated.

5.1 Why DNA?

The unique physical, chemical and informational properties of DNA make it the 'main molecule' not only in biology, but potentially in nanotechnology too. The Watson-Crick binding between DNA base pairs allows the replication and the local interaction geometry at sticky ends provides self-assembly. The cloning of DNA and *in vitro* evolution toward optimisation can be achieved through amplification by PCR and selection. The information storage density in DNA is extremely high. DNA could serve as a template for molecular electronics.

Dead DNA (structural DNA)		BBa_J35000 - BBa_J35399
Structures	DNA origami	BBa_J35000 - BBa_J35099
	protein binding parts	BBa_J35000 - BBa_J35029
	aptamers	BBa_J35030 - BBa_J35059
	others	BBa_J35060 - BBa_J35089
	others	BBa_J35090 - BBa_J35099
Devices	tweezers	BBa_J35100 - BBa_J35199
	nanomechanical switches	BBa_J35100 - BBa_J35119
	nanoactuators	BBa_J35120 - BBa_J35139
	walking nanomachines	BBa_J35140 - BBa_J35159
	others	BBa_J35160 - BBa_J35179
	others	BBa_J35180 - BBa_J35199
Systems		BBa_J35200 - BBa_J35299
Others		BBa_J35300 - BBa_J35399
Alive DNA (protein-coding DNA)		BBa_J35400 - BBa_J35799
Parts		BBa_J35400 - BBa_J35499
Devices		BBa_J35500 - BBa_J35599
Systems		BBa_J35600 - BBa_J35699
Others		BBa_J35700 - BBa_J35799
Other DNA		BBa_J35800 - BBa_J35999

Fig. 3 Extended BioBricks repository for DNA nanotechnology



Fig. 6 Freiburg 2006 iGEM team and logo

main creative principle is the ‘learning by doing and doing by learning’.

6 Future perspectives

We consider DNA nanotechnology to be a field tightly linked to synthetic biology and artificial life, including: (1) direct DNA synthesis, (2) abstraction, and (3) development of standard parts to promote open tools and databases. Our experiences help us to recognise that molecular quantum-dot cellular automata may be a solution for the next generation of fast computing devices, which could operate in inorganic and organic environments [41]. We use a DNA origami technique with 6 nm scale resolution to fabricate these devices. Our project combines DNA database development and simulation with DNA experimentation.

As mentioned before, the basic idea was to design DNA such that it folds into a DNA sheet, which we called an addressable platform with 6 nm scale resolution. A specific pattern of molecules is then attached. These molecules could be sensors, logic gates or actuators. We designed

DNA sheets and turned them into different 3D shapes to form nanoobjects like the tetrahedron. These nanoparticles with molecular sensors, logic gates and actuators can be considered candidates for a massive parallel computation. One could define local interaction rules for these molecular agents and perform a distributed amorphous computation. Agent based modeling will be used to simulate the behaviour of the smart nanoparticles.

Yet we need to develop our state-of-the-art systems. We shall concentrate our future efforts on: (1) the design of aptamers to QDs from different materials, which should be suitable for molecular QCA; (2) the development of DNA origami wiki-based database; and (3) the hierarchical design of 3D DNA nanostructures. We consider unconventional computing, cryptography, nanoelectronics, nanooptics, nanosensors, drug delivery systems and smart nanomaterials as potential applications of the DNA origami technique in the near future.

7 Conclusions

Our DNA-folding project is not a typical synthetic biology project, because we experimented with structural DNA, not DNA coding proteins. We are going to merge the DNA origami static structures and the dynamic DNA BioBricks constructs to design living machines. Following the ethics of artificial life, we will try to pump some aesthetic principles and rules (symmetry, periodic patterns and recursion) into our future creations as a kind of control for these systems. At the start of the project we made efforts on the addressable high resolution platform that will allow us the bottom-up fabrication of complex nanostructures by self-organisation. The spatial resolution of 6 nm serves each insertion into an oligonucleotide staple as a 6 nm pixel. The main possibility is that a long, single strand of DNA can be folded arbitrarily into 2D or 3D shapes using a raster fill technique. DNA can then be programmed to form larger assemblies, including extended periodic lattices.

In the second step of our project we will concentrate more on the algorithmic self-assembly of 3D DNA tiles. Smart nanoparticles, which contain sensors, logic gates and actuators, will allow us to perform a distributed amorphous computation in the third phase of the project. The computation will take place between neighbours. Tiny ‘computational agents’ will interact with their nearest neighbours and swap information. We could directly program the molecular dynamics and control these smart particles. We should estimate how much computing resources it requires. How complex should the computation be? How many computing components? All of these questions are deeply theoretical. To avoid great difficulty we will simulate these smart nanoparticles using virtual and real objects. We will also use the Breve simulator [42].

In the future, the designed fragments of DNA/RNA could be seen as a type of genetic program and a part of a ‘genetic robot’. It would be needed to add data processing, memory storage and communication elements to produce nanoagents that could operate in cells. Theoretically, these genetic programs could be introduced into and replicated by living cells in order to control their processes. An assembly of these programs could be installed as transgenes within the body, coordinating with homeostasis [43], for diagnostic, effective treatment, and to extend the internal possibilities.

Although we have done a lot of designing, we have repeated the first Rothmund experiment (rectangle) only recently. We have no idea whether it works well for 3D structures. Until we can do them, we have no idea

whether they work well. We have no idea whether RNA origami will work *in vivo*. We need to perform many experiments. The successful development of our project depends dramatically on the strong financial support and the official status of this project. We are only a start-up team, who needed to be encouraged to express the opportunities. At the moment, our research is mostly theoretical, although we are looking for the best applications of this technology. We tried to do a project involving the latest findings in computer science, physics and biotechnology. I ask myself, what will a computer be by 2050; what about the ultimate laptop and embedded computer? These questions lead me to remember Stephen Hawking who warned: 'Alter our DNA or robots will take over'.

8 Acknowledgments

The Freiburg 2006 iGEM team and logo are shown in Fig. 6. The author wishes to thank many people for help, friendly criticism and support, especially Irina Petrova, Mona Klein, Yutthaphong Phongbunchoo, Konrad Divon, Tamara Ulrich, Svetlana Santer, Andreas Komnik, Marya Lieberman, Paul Rothmund, Peixuan Guo, Nadrian Seeman, and Jan Korvink. Sorry to those not on the list.

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Engineered human cells: say no to sepsis

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Abstract: Mammalian systems can be a subject of cellular engineering in a similar way to bacterial cells. Our team decided to modify the existing mammalian cell signalling network of the innate immune response to bacterial infection. Binding of bacterial components (pathogen associated molecular patterns, or PAMPs) to a family of Toll-like receptors (TLRs) activates the cells of the immune system but an exaggerated response may lead to systemic inflammation and sepsis which is often fatal. We designed a feedback loop, which inhibits the signalling cascade at the weak spot – protein MyD88 – which is the consensus adaptor protein of the surface-expressed TLRs. A mathematical model of cell activation with an engineered feedback loop predicts a decrease of cellular activation after repeated stimulation of TLR. We have prepared 26 BioBricks for mammalian systems and deposited them into the BioBrick Registry at MIT. Mammalian cells transfected with the feedback loop construct performed as designed with a decrease in the cellular response upon repeated stimulation with PAMPs. Cell activation decreased without completely abolishing the responsiveness to the bacterial stimulus, therefore our engineered system represents an artificial type of immunotolerance. The Slovenian team was composed of seven undergraduate students of microbiology and biochemistry and five mentors from the National Institute of Chemistry and University of Ljubljana Faculty of Chemistry and Chemical Technology.

1 Aims of the project

The aim of our project was to apply the principles of synthetic biology to the area of innate immune response. Toll-like receptors (TLRs) at the cell surface detect the presence of bacterial components (pathogen associated molecular patterns, or PAMPs) and activate the innate immune response [1]. This response is essential for survival in an environment containing pathogenic bacteria. However, the response to infection, triggered by TLRs, amplifies itself and when this becomes excessive, can lead to serious consequences such as sepsis and even death [2]. The number of casualties is estimated to exceed 200 000 each year within the European Union alone. The specific idea of our project was to introduce a feedback mechanism into the TLR signalling pathway, which would decrease the exaggerated response to a persistent or repeated stimulus with PAMP, without completely shutting it down, thus retaining the host's defence against invading bacteria. Inhibition of the exaggerated response could be achieved

if the activation of the immune response by PAMPs would also trigger the expression of an inhibitor that would inactivate the signalling pathway. In our project, we used a dominant negative mutant of MyD88 [3], dnMyD88, placed under the NF- κ B responsive promoter, which is activated after stimulation with PAMPs. Inhibition of the signalling pathway also shuts off the production of the inhibitor, therefore the responsiveness of the system is restored once all the inhibitor has degraded. The lifetime of the dominant-negative inhibitor dnMyD88 could be additionally modulated by a tag facilitating a rapid degradation (PEST sequence).

This idea is similar to the natural mechanism of tolerance already present in mammalian cells and which decreases the response to repeated bacterial stimulation. This natural tolerance is activated slowly and operates through several different mechanisms (e.g. IRAK-M, downregulation of receptors etc.) [4]. Our feedback mechanism (i.e. artificial tolerance) should decrease the time of tolerance induction and 'attack' the signalling pathway in a way which has not been used in the natural system.

The adapter protein MyD88, consisting of a TIR and a death domain, is at the crossroads of Toll-like receptor signalling. Therefore, activation of surface-expressed TLRs recruits MyD88 to the cell membrane, which subsequently attracts signalling kinases through its death domain. Downstream signalling then proceeds through protein phosphorylation, association and this ultimately leads to the translocation of transcription factor NF- κ B into the nucleus, which can directly or indirectly affect transcription of more than 1000 different genes [5]. As an experimental system, we selected receptor TLR5 with its agonist flagellin and receptor TLR4/MD-2 stimulated by lipopolysaccharide (LPS). However, our system is designed to work with any signalling receptor that has MyD88 in its signalling pathway (i.e. TLRs 1, 2, 4, 5, 6, 7, 8, 9, 11, or IL-1), therefore our approach could

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in principle work also for chronic inflammatory diseases, which require signalling through the MyD88 adapter protein, such as gout, atherosclerosis and so on [6, 7].

2 Description of the work

2.1 Parts design

Essentially, all BioBrick parts were designed *de novo*, since no parts such as eukaryotic promoters, terminators, desired proteins or their domains for signalling pathway modification, degradation flags and reporters had previously been designed and deposited in the Registry. Primers for parts amplification included restriction sites for cloning into BioBrick vectors with ccdB domain, which simplified cloning. To streamline the BioBrick parts assembly, we designed a special BioBrick-based vector containing a eukaryotic terminator. All our composite parts (promoter plus part) were cloned into this vector.

For fusion proteins (e.g. dnMyD88-linker-rLuc-linker-PEST), parts were designed as basic parts (not composite), although they are fusion proteins. Linkers were introduced using PCR Overlap Extension method. These parts were combined with the respective promoters (CMV^P and NF- κ B^P) by BioBrick assembly technique.

2.2 Experiments on mammalian cell cultures

Functionality experiments were performed in human embryonic kidney cells (HEK293). Cells were simultaneously transfected with vectors coding for suitable TLRs, which are not present at the surface of HEK293 cells and, if necessary, reporter luciferases to detect the cell activation.

2.3 Detection systems

We expected the expression of the dominant negative protein dnMyD88 to block the signalling pathway, downregulating the transcription of inflammatory proteins. The constructs we prepared were designed for various detection systems, including flow cytometry, ELISA and luminescence measurements of reporter proteins.

We detected activation of the protein phosphorylation cascade with anti-phosphoprotein antibodies, specific for the phosphorylated form of ERK kinase (pERK). Phycoerythrin-labelled secondary antibodies were used for detection by the flow cytometer.

Several composite BioBricks parts (e.g. NF- κ B^P-fLuc, CMV^P-rLuc, NF- κ B^P-dnMyD88-linker-rLuc-PEST and NF- κ B^P-dnMyD88-linker-rLuc) were designed for the detection of transcriptional activation by means of luminescence of reporter proteins. Two different enzymes were used in these analyses: the firefly luciferase (fLuc) and the *Renilla* (sea pansy) luciferase (rLuc). Since they can discriminate between their respective bioluminescent substrates and do not cross-activate, we could measure the activity of both luciferases in one sample in a so-called dual luciferase assay [8]. Typically, the experimental reporter, NF- κ B^P-fLuc was correlated with the effect of specific experimental conditions, while the activity of the co-transfected 'control' reporter gene CMV^P-rLuc provided an internal control. Normalising the experimental reporter gene to the activity of an internal control minimised the variability caused by differences in cell viability and transfection efficiency. Thus, dual reporter assays allowed for more reliable interpretation of the experimental data by reducing the extraneous influences.

Influence of composite parts NF- κ B^P-dnMyD88 and NF- κ B^P-dnTRAF6-linker-GFP on innate immune response pathway was also monitored by ELISA, designed to detect free NF- κ B transcription factor.

3 Organisation of the team and work

The student team was composed of seven undergraduate students of microbiology (six) and biochemistry (one) from the Biotechnical Faculty and Faculty of Chemistry and Chemical Technology of the University of Ljubljana, Slovenia, respectively. Members were selected among 34 candidates from various faculties based on the candidates' essays on project proposal and technical solution of the problem, their grades and CV. Among the 14 candidates interviewed by the supervisors, seven members of the team were selected (Fig. 1). In addition to the two supervisors, support was provided by three mentors from the Laboratory of Biotechnology of the National Institute of Chemistry, who were supervising different detection systems, constructions of different groups of BioBricks and the progress of the project.

From the short-listed project ideas, discussed in April, the final project topic was decided upon in May. Experimental work started in June and lasted with increasing pace until the end of October.

4 Mathematical model construction

The signalling pathway of pathogen-associated molecules (PAMPs) through TLRs utilising the MyD88-dependent signalling pathway was modelled using CellDesigner [9]. Intracellular activation cascade was represented in a simplified way, collapsing into a single stage the steps between the association of MyD88 with activated TLRs and translocation of NF- κ B into the nucleus, since these steps were not different in the original and engineered signalling pathway (Fig. 2). Insertion of the dnMyD88 gene under the NF- κ B-responsive promoter was added to the original pathway with the resulting dnMyD88 being able to bind to the activated TLRs with the same kinetics as the wtMyD88 but resulting in a dead-end complex. Simulation of the kinetics of cellular responsiveness to the TLR agonists was performed with two pulses of agonists (as later performed in the actual experiment) and it resulted in the expected properties of the response with the first pulse inducing the transcriptional activation of the NF- κ B responsive genes (inflammatory mediators) and also dnMyD88. Stimulation with the second pulse produced substantially lower activation due to the presence of cytoplasmic inhibitory form of MyD88, competing with wtMyD88 for binding to the activated TLRs. Analysis of the simulation showed the effect of the lifetime of the inhibitor on the relaxation of the system's responsiveness to the original level.

5 Experimental results

We have tested cell activation by flow cytometric detection of intracellular phosphorylation and active NF- κ B-based ELISA, however dual luciferase reporter system was selected as the most reliable and sensitive method. We tested the functionality and expression of the part containing NF- κ B-dependent promoter in a construct with dnMyD88-rLuc (BioBrick part: NF- κ B^P-dnMyD88-linker-rLuc). Stimulation of TLR5-transfected HEK293 cells with their agonist flagellin induced translocation of



Fig. 1 Proud team members with the BioBrick award after the announcement of the Grand Prize winner in the MIT Kresge Auditorium

First row: Matej Skočaj, Jernej Kovač, Alja Oblak, Marko Dolinar

Second row: Monika Ciglič, Jelka Pohar, Roman Jerala, Ota Fekonja, Rok Tkavc and Gabriela Panter

Mateja Manček Keber and Mojca Benčina, two of the mentors of the Slovenian team, could not attend the jamboree and are missing from the team picture, which was taken by the iGEM ambassador Robin Künzler

the NF- κ B into the nucleus and upregulated transcription of NF- κ B responsive genes. *Renilla* luciferase activity was detected only in stimulated cells with significant activity appearing from two hours after the cell stimulation, which

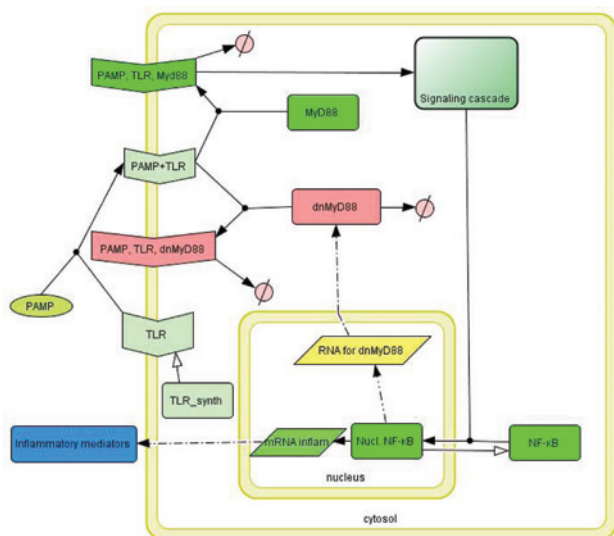


Fig. 2 Model of TLR signalling with the engineered negative feedback loop (red elements in the diagram) for the inhibition of MyD88-dependent TLR signalling

Binding of PAMP to TLR causes its activation and binding of MyD88, which triggers the signalling pathway or inhibitory dnMyD88, resulting in a dead-end complex

proved the functionality of the construct (i.e. promoter inducibility).

We also measured NF- κ B-responsive firefly luciferase activity of our artificial tolerance system. A decrease of activity was expected after the amount of time sufficient for the synthesis of inhibitory fusion protein in cells. To our disappointment, no appreciable decrease of cell activation was observed. One of the possible reasons for the lack of inhibition of the dnMyD88-rLuc protein fusion could be sterical hindrance of dnMyD88 by the C-terminal rLuc fusion partner. The dnMyD88 interacts with the TIR-domain of TLRs in direct proximity to the cell membrane. The fusion of this part with a larger protein domain could thus prevent the interaction, particularly since the wt MyD88 has the TIR-domain at its N-terminus fused to the death domain. Based on these observations we conducted our experiments with a device containing dnMyD88 without any additional protein domain. In the following experiment, cells were co-transfected with TLR5, reporter plasmids (NF- κ B^P-fLuc and CMV^P-rLuc) and our feedback loop, BioBrick part NF- κ B^P-dnMyD88. We have designed the experiment by two consecutive pulses of TLR stimulation separated by 4-6 h, which is sufficient time to accumulate the inducible inhibitor. Control cells should respond to both stimuli, while the cells with our 'inhibitory feedback loop' should respond to the same extent only to the first pulse. Indeed, our results show that the system with inducible dnMyD88 responds weaker to the second stimulus of flagellin (Fig. 3). The minimal delay between the two stimuli corresponds to the time required for the synthesis

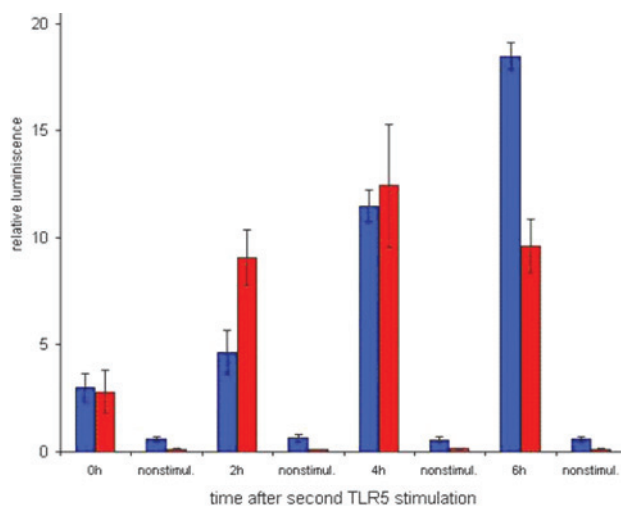


Fig. 3 *dnMyD88* feedback loop decreases cellular activation by repeated stimulation with bacterial flagellin

Normalised luciferase activity (fLuc/rLuc) of cells stimulated by the addition of two pulses of flagellin separated by 6 h is shown. HEK293 cells were transfected with the device comprised of NF- κ B^l-inducible dnMyD88 (red) and compared to wild-type cells (blue, transfected only with TLR5 and reporter plasmids) and stimulated in parallel.

of the inhibitor as predicted, since it does not completely abolish the cellular response but restores it after an extended delay following the stimulation.

6 Conclusions

- The principles of BioBricks used in bacteria and yeasts can also be used in mammalian cell systems.
- We have successfully implemented a feedback loop that decreased the cellular activation through the MyD88-dependent signalling pathway.
- The response of inhibitory feedback loop is transient and the cell responsiveness is restored after the synthesised inhibitor has degraded.
- dnMyD88 inhibitor was not effective as fusion with luciferase or GFP at its C-terminus, which may be due to the steric hindrance; it is likely that the N-terminal fusion of reporter domains or, alternatively, domains with longer linkers would be functional as signalling inhibitors.
- Our system, mimicking the natural mechanism of tolerance, can be activated within a couple of hours and may

be beneficial to prevent excessive stimulation, which could lead to a chronic or acute inflammation.

- The simplified model of the TLR signalling qualitatively captures most of the features of the natural system.

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Brainstorming biology

S. Dabholkar and M. Thattai

Abstract: The NCBS iGEM 2006 team grew out of a three month interdisciplinary workshop on synthetic biology, built around teamwork and brainstorming. Our emphasis was on the development of a design process, not merely on a successful outcome. We worked on three projects: (1) converting a transient stimulus to a sustained response; (2) two-dimensional regulation of bacterial chemotaxis; and (3) synchronising bacterial cell cycles. Common to all these projects was the idea of modifying an existing complex biological system in order to achieve some desired behaviour. Here we share our experimental successes and failures, the lessons we learned about the practice of synthetic biology, and our thoughts about the special role synthetic and open-source biology might play in India.

1 The workshop

When a group of us at NCBS sat down to organise a workshop on synthetic biology, we were energised by the idea that we were helping to create a global community, participating in the first truly international iGEM. We also wanted the workshop to represent a very different way of doing science. It was time-bound, in that we wanted to be able to design, construct, and validate novel genetic networks, all within a three-month period. It was interdisciplinary in that our participants included students and faculty from various scientific disciplines, researchers from industry, and even a journalist and a lawyer. And it was a team effort in that at each weekly brainstorming session, student groups would present their experimental results, and all participants would provide feedback and fresh ideas. In many ways it was we, the participants, who were the real experimental subjects, not the bacterial cells in our Eppendorf tubes. Could we find ways to pool our skills, develop a design process, and successfully generate a functional synthetic construct?

2 The many successes of synthetic biology

The first step was to introduce the participants to the idea of synthetic biology. We soon realised that ‘synthetic biology’ has actually been going on for quite some time. In coming up with our own design ideas, we could draw inspiration from previous projects. So we asked: ‘What were the common characteristics of such projects, and which ones represented synthetic biology success stories?’

There are actually two varieties of synthetic biology. First, ‘conservative synthetic biology’ involves taking some complex biological process and modifying it slightly, standardising it, and making its use widespread among the research community. There are plenty of examples, but here are five: (1) restriction digestion, finding restriction enzymes for a variety of sequences; (2) homologous

recombination, which is the workhorse of cloning; (3) PCR: a brilliant idea to use a polymerase which works at high temperatures to amplify small quantities of DNA; (4) monoclonal antibodies, standardised and reproducible; and (5) RNAi, using small RNA fragments to shut down genes in a sequence-specific fashion. It is safe to say that in none of these cases do we deeply understand the underlying mechanism: we could not have come up with these from scratch, we simply appropriated them for our own purposes. In contrast, ‘ambitious synthetic biology’ involves actually reconstituting a biologically inspired function from the bottom-up. For example, there are attempts to make artificial membranes which have some of the important characteristics of biological membranes, actively transporting molecules, growing and dividing. One of the few successes of ambitious synthetic biology is DNA sequencing. The ability to read an arbitrary sequence does not exist in nature, but was achieved artificially after a great effort spanning decades. It does not involve any machines to be found in the natural world, but instead is heavily dependent on unnatural molecules invented by chemists.

3 The projects

We decided to go conservative. We therefore came up with network designs that exploited an existing complex biological system, into which we introduced small modifications in order to achieve some desired behaviour. We finally settled on three design ideas, based on three existing biological properties. Because the time was limited, we chose projects that involved varying levels of difficulty: the first two were relatively simple and workable, while the third was rather ambitious.

3.1 Bistability: converting a transient stimulus to a sustained response

Bistable systems are toggle switches that, once thrown into an ON or an OFF state, maintain that state indefinitely. The lactose utilisation network in *Escherichia coli* exhibits bistability under certain conditions [1]. In contrast, other types of systems exhibit transient responses. For example, the SOS system of *E. coli* produces a limited pulse of transcription in response to UV exposure [2]. We asked whether the bistable *lac* system could be used to convert a transient

pulse of UV exposure into persistent GFP expression (Fig. 1a).

We used a plasmid to express the permease lacY under the control of a UV-sensitive SOS promoter. This construct was placed in a strain of *E. coli* in which GFP was chromosomally expressed from the *lac* promoter. The cells were grown in a medium containing TMG, an inducer of the *lac* promoter that is transported by lacY. In principle, UV exposure would trigger transient expression of lacY, and the resulting TMG uptake would turn on *lac* expression. In practice, the design did not function as expected. We soon realised that the presence of extraneous lacI binding sites on the plasmid backbone had perturbed the system, destroying its natural bistability. It was an important lesson for us in designing synthetic networks, that unforeseen interactions with the host could corrupt even the simplest systems.

3.2 Gradient-sensing: two-dimensional regulation of bacterial chemotaxis

The ability of *E. coli* to sense and move up or down chemical gradients is known as chemotaxis, and is achieved by coupling membrane receptors for various chemo-attractants or chemo-repellants with the flagellar motor system. We reasoned that cells could be made to selectively respond to certain chemical gradients by specifically up- or down-regulating the relevant receptors (Fig. 1b).

We started with *E. coli* strain UU1250, which has its four major chemotaxis receptors knocked out [3]. We re-introduced two of these: the aspartate-sensitive Tar receptor, and the serine-sensitive Tsr receptor, under independently inducible promoters. In parallel, we designed a three-gradient system consisting of two perpendicular serine and aspartate attractive gradients, with a third

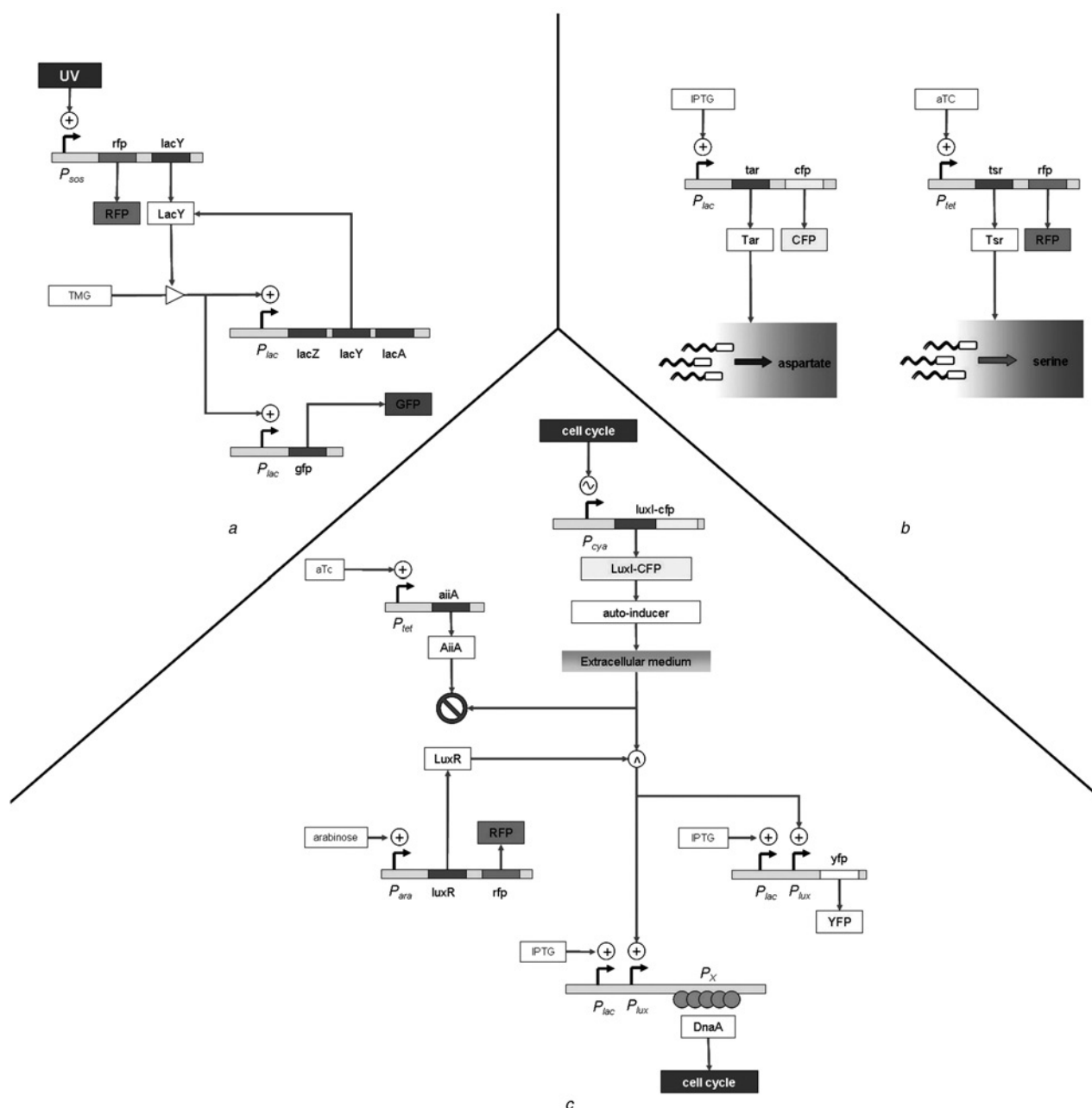


Fig. 1 Meet our networks

- a Bistability: converting a transient stimulus to a sustained response
- b Gradient-sensing: two-dimensional regulation of bacterial chemotaxis
- c Oscillations: synchronising bacterial cell cycles

default repellent gradient at a 135° angle to these. Positive linear combinations of these three gradients can generate any vector on a two-dimensional surface. However, implementing such a gradient turned out to be far from trivial. Moreover, we were not able to rescue chemotaxis in the knockout strain.

3.3 Oscillations: synchronising bacterial cell cycles

Populations of oscillators that are weakly coupled tend to synchronise. Mathematical models have suggested that such a coupling between cells can be achieved by using the *Vibrio harveyi* quorum sensing machinery [4, 5]. However, in order to achieve synchronisation it is essential to have a fairly reliable oscillator. For this purpose, we turned to the cell-cycle itself (Fig. 1c).

To generate an oscillatory signal, we used the adenylate cyclase promoter P_{cya} , whose expression oscillates as the cell cycle progresses, even when plasmid-borne [6]. The next step was to transmit this signal from cell to cell, for which we used the *Vibrio* LuxI-LuxR system. The enzyme LuxI drives the synthesis of AI, a chemical that diffuses across the cell membrane; LuxR is an AI-dependent transcriptional activator. We expressed a LuxI-CFP_{LVA} fusion protein from P_{cya} , and expressed YFP_{LVA} under the LuxR-dependent P_{Lux} promoter. Thus, CFP fluorescence would report if oscillations were being generated and transmitted, while YFP fluorescence would report if they were being received. As a non-oscillating control, we introduced RFP under a cell cycle-independent arabinose promoter. The final, most difficult step was to use the received oscillations to affect cell-cycle progression. For this, we turned to DnaA, a protein which regulates the initiation of DNA replication, and is therefore the central cell cycle regulator *E. coli* [7]. Between replication events, DnaA is sequestered onto chromosomal binding sites known as DnaA boxes, thus reducing its cytoplasmic concentration. If we were able to modulate the sequestration of DnaA, we would be able to speed up or slow down cell cycle progression. Our strategy was as follows. Investigations of transcriptional roadblocks have suggested that a DNA-bound protein can be knocked off by actively transcribing RNA polymerases [8]. We introduced DnaA boxes downstream of a P_{Lux} promoter so that increased AI concentration would drive transcription through these boxes, releasing DnaA into the cytoplasm and allowing the initiation of DNA replication.

This was the largest genetic construct that we used, consisting of 18 existing Biobricks and three newly generated Biobricks. We introduced three inducible promoters as experimental knobs that could be used to tune the network's behaviour, and three fluorescent reporters to monitor its dynamics. Key to the design was modularity: the entire system consisted of an oscillatory module, a transmission-receiver module, and a cell-cycle regulatory module. Coupling these modules combinatorially allowed us to test various aspects of network function, with the following results. First, we found that P_{cya} indeed produces an oscillatory output, as demonstrated by oscillations in CFP fluorescence (Fig. 2a). Second, the LuxI-CFP_{LVA} fusion is functional, generating AI and driving the oscillatory expression of YFP (Fig. 2b). Interestingly, the peak-to-trough ratio of YFP oscillations was greater than that of CFP oscillations, possibly due to non-linearity via LuxR regulation. As expected, the RFP control signal did not oscillate (Fig. 2c). However, synchronisation could not be achieved because the presence of DnaA boxes did not seem to affect cell-cycle progression. In spite of

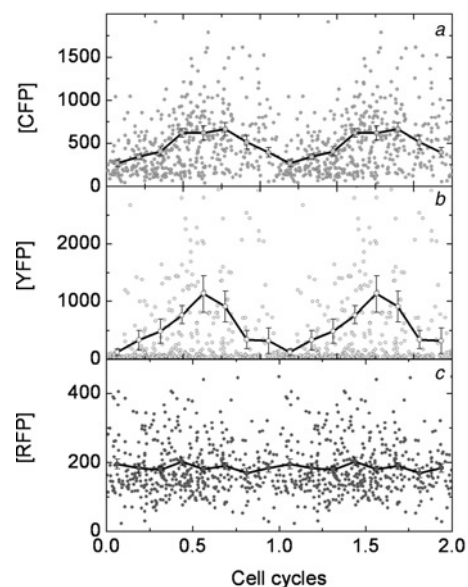


Fig. 2 Transmitting and receiving oscillatory signals

a CFP fluorescence indicates oscillatory expression from P_{cya}
b YFP fluorescence shows the successful propagation of oscillations through the *Vibrio* quorum-sensing system
c RFP fluorescence is used as a non-oscillating control
 In each panel, every datapoint represents a single cell. Fluorescence is plotted vertically, and cell cycle phase (inferred using cell length) is plotted horizontally. The data is shown in duplicate, to emphasise its periodic nature over two cell cycles. Dark lines show mean and standard error, calculated by binning

this failure, this proved to be our most interesting and illuminating project, as it taught us a great deal about the process of network design and testing.

4 Synthetic biology in India

In parallel to the benchtop experiments, we used the brainstorming sessions to explore various aspects of synthetic and open-source biology, especially focusing on the Indian context. Vishwas Deviah, a lawyer working on patent issues, taught us about the Indian patent regime, about open-source licensing, and about the pitfalls associated with patenting living organisms. P. Babu, Kunj Tandon, and Shashi Thutupalli, all research scientists working in industry, told us about the business models of several emerging Indian biotechnology and pharmaceutical companies, and explored the links between synthetic biology, innovation, and entrepreneurship. Anupama Bhat, Neelam Agarwal, and Varun Mishra, all working at a Bangalore-based life sciences company, speculated whether the transparent, community-based approach of open-source biology might be used to address problems specific to the developing world, reducing the cost of biological information, improving our understanding of tropical diseases, and addressing conservation issues.

Our synthetic networks might not have performed as well as we'd hoped, but the workshop itself was an unqualified success. Synthetic biology seems to have hit a sweet spot, capturing and holding the interest of participants from diverse backgrounds, over three full months. The workshop was a unique forum, a common space in which experts from industry and academia could come together each week to discuss and implement ideas on innovation and entrepreneurship. Building on this year's success, we plan to make this an annual event at NCBS, and hope to



Fig. 3 The NCBS iGEM 2006 team

Hiding in the back: Sugat Dabholkar; standing, left to right: Adil Ghani Khan, Mukund Thattai, Ashesh Dhawale, Dhanya Parameshwaran, Krithiga Sankaran; kneeling, left to right: Aparna Suvrathan, Ruchi Malik

replicate it at other undergraduate and graduate-level institutions around the country. The prospects are immensely exciting.

5 Postscript: Notes from the benches

As members of the NCBS iGEM 2006 team (Fig. 3), we knew this was a chance to think different. We were a rather diverse group of graduate students, with backgrounds ranging from neuroscience to developmental biology. For most of us, the idea of synthetic biology was completely unfamiliar, and therefore a chance to learn something new. The first thing we learned was that synthetic genetic networks were relatively easy to build – the hard part was coming up with the designs. During our brainstorming sessions, we came up with many crazy ideas: all were interesting, some were ambitious, others less so. We learned a great deal about how to mathematically model such networks, and used these models in the process of selecting and improving our designs. And of course, we put in many hours at the bench, trying to get our constructs to behave the way they were supposed to. This turned out to be more difficult than we had anticipated – some things worked, most things didn't. But in the process, we did figure out how to build a chemostat, how to make custom chemical gradients, how to grow biofilms and how to track oscillations in single cells. And, most importantly, we got to present our results, both the successes and the failures, at the iGEM Jamboree. Meeting the other teams and seeing the difficulties that they, too, had faced, we realised that this is still a very young field, with a lot of work to be done. So will this approach ever work? Will we someday be able to design and construct genetic circuits to order, within a few weeks or months? At least some of us wish we were undergrads

again, so we'd have the time to find out! But for us grad students, it's time to get back to work!

6 Acknowledgments

A great many people contributed to making this workshop a success. We would particularly like to thank: Reshma Shetty, our iGEM Ambassador; Upi Bhalla, Mitradas Panicker, and Guhan Jayaraman for great discussions and design ideas; Senthil and Durga for all the help at the bench; and Biswajit Roy of Bangalore Genei for custom cloning services. Our projects were fully funded by NCBS.

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Development of an inducible three colour bacterial water colour system

T. Brown, C. Chang, B. Heinze, P. Hollinger, J. Kittleson, K. MacDow, D. Reavis, J. Curry and M. Riley

Abstract: Here we present the work of the 2006 University of Arizona team in the iGEM (international Genetically Engineered Machine) competition sponsored by MIT. Our aim was to develop an inducible water colour system for painting bacteria in three colours. Using BioBricks, a plasmid was designed for insertion into *E. coli* which would allow the bacterium to respond to each of three different chemical inducers by producing yellow, cyan or red fluorescent proteins. In principle the inducers could be deposited on a bacterial lawn using a high-resolution printer so that three colour images could be produced with a spatial resolution matching the size of the bacterium. This could be the first step towards producing a bacterial lawn that behaves like a canvas for watercolour painting or eventually a three-colour television set. The application connects with current interest in precise control of cellular response desired in biosensors and bioengineered materials. The University of Arizona iGEM team called the 'Cell Raisers' was assembled in May 2006 and worked enthusiastically throughout the summer. The team was comprised of six undergraduates (Tyler Brown, Brian Heinze, Patrick Hollinger, Josh Kittleson, Kevin MacDow, and Dan Reavis), one graduate student (Carlos Chang), and two faculty members (Joan Curry and Mark Riley). This proved to be an ambitious project, and while the final goal was not fully realised, first steps were made in terms of design, plasmid construction and bacterial deposition with an inkjet printer. Patrick Hollinger and Brian Heinze gave the technical presentation to judges and competing teams on Saturday, November 4, 2006 in MIT's Stata Center. The team also presented a poster that was viewed in the evening after all the presentations. For their efforts, the University of Arizona team received honourable mention with special consideration: 'For progress toward synthetic biology in three colours'.

1 Aims of the project

Our overall aim was to develop an inducible water colour system for painting on bacteria in three colours. Specific goals were (1) to create a colour image utilising bacterial fluorescence and (2) to develop a control scheme that will allow for selective activation of bacteria by precise stamping or introduction of inducers or through precise placement of bacteria to produce complex images. Substantial progress was achieved towards both goals by the University of Arizona Cell Raisers. The team logo is presented in Fig. 1.

2 Description of the work

A number of project ideas were considered and substantial time was invested in project design. The initial focus was on developing a plasmid which would allow for the activation of fluorescence by the placement of different chemical inducers. As detailed in Table 1, the team identified and selected three independent operons to control expression of three different fluorescent proteins.

A plasmid was constructed from BioBricks available from the Registry of Standard Biological Parts (http://parts.mit.edu/registry/index.php/Main_Page) provided by MIT as a component of the iGEM competition. Using the

standardised restriction enzyme sites and preconstructed bricks, the Lac repressor (LacI), Tet repressor (TetR), and HSL inducer (LuxR) were attached to constitutive promoters and high output ribosome binding sites. Each control element was then ligated to the associated fluorescent expression element. The expression elements were previously ligated to promoters under the control of the repressors and inducers as designated in Table 1. The assembly process was carried out using a parallel assembly to generate composite parts as presented in Table 2. The plasmid was transferred into *E. coli* using standard methods. DNA sequencing was performed to confirm that the plasmid was constructed correctly.

Simultaneously with the plasmid construction experiments, team members worked to develop methods for the application of inducers via a controlled mechanism to achieve maximum image resolution. Several inducer introduction methods were evaluated for their ability to deliver a precise spatial arrangement on bacterial lawns grown in a standard Petri dish. The first, and least precise approach, was to apply inducers by pipetting by hand and allowing the inducers to seep into the agar. Initially, the bacterial cultures were grown on LB + ampicillin in inducer concentrations ranging from no inducer to $10\,000 \times K_m$. All three chemical inducers were applied to the cells. However, no quantifiable difference was found between induced and non-induced bacteria. The initial assessment was: strong expression of yellow, weak expression of red, little to no expression of cyan.

Specific colonies grown in the initial cultures were selected for strong expression and grown individually.



Fig. 1 University of Arizona team logo for the 2006 iGEM competition

Table 1: Summary of project design

Regulator	Type	Controlled by	Colour
LuxR	Positive	Homoserine lactone (HSL)	Red fluorescent protein (mCherry)
TetR	Negative	Anhydrotetracycline (AHT)	Cyan fluorescent protein
LacI	Negative	Isopropyl β -D-1-thiogalactopyranoside (IPTG)	Yellow fluorescent protein

Fig. 2 shows single genotype cultures showing differential expression of fluorescent proteins. Initial sequencing verified the presence of all three fluorescent genes in the final construct.

Control over the construct was essential. Variable phenotypes due to the original construct made control of fluorescent protein expression impossible. By revisiting intermediate constructs and using strains containing constitutively expressed mCherry and yellow fluorescent proteins, the focus of the project shifted towards development of maximal image resolution through various mechanical methods.

Precise addition of the inducers was not successful as the bacterial response to inducers required an overnight cultivation, during which time bacteria reproduced. This produced blurred spatial resolution due to both colonial growth and bacterial migration. Fig. 2 shows photomicrographs of the resultant structures. The image in the top left corner shows two bacterial colonies presenting a non-uniform distribution of fluorescence. Interpretation of these images is a challenge, however, the most likely explanation for the structures shown is that the bacteria within individual colonies are motile within a colony and so spread outwards from an initial seed cell. Not all colonies show a high level of fluorescence. Most often fluorescence appears in only half of a

colony, suggesting an instability in the constructs as the bacteria replicate.

The first idea to improve the resolution and standardisation of the fluorescent proteins was to place inducers on the media by means of an inkjet printer. The inducer solution would be printed on a thin transfer sheet (such as standard or heavy card stock paper). The transfer sheet is then placed on the media to make contact between the cells and inducer. Effectively, the inducer could be placed in any given arrangement to produce a picture and in any given amount. This method was also modified for the application of cells.

We developed a related, but more precise method, using a mold generated through rapid prototyping from a computer-aided design file. This stamp was to be used to deliver inducer only on the positive, raised portions of the stamp.

Fluorescence was inconsistent and there was no clear correlation with the applied inducers. Single colonies exhibited strong fluorescence in yellow, weak fluorescence in mCherry, and very little to none in cyan channels. The origin of the problem was unclear and due to time limitations it was not possible to complete a systematic study of the plasmid operation and inducer effectiveness. This remains for future work.

Based on these difficulties, we revisited our intermediate constructs and evaluated a more exacting method for placing cells using an inkjet printer such as an HP Officejet K550 printer. This printer was selected due to its independent ink cartridges which could more easily be loaded with separate bacterial solutions than most similar printers. The concept was to replace the ink with bacterial cells which had been induced to present specific fluorescent colours (blue, red, yellow). A standard syringe and needle was used to remove ink, rinse the cartridges, and to add bacteria (Fig. 3). The ink delivery lines were primed with cells by injecting through the print head septum. The cartridges were rinsed multiple times with de-ionised water. Transformed cells were scraped from agar plates, added to de-ionised water, and mixed. This bacterial solution was then used to refill the cartridges. The lines running from the cartridges to the print heads were primed with the cell solution via syringe at the print head septum.

The primary challenges associated with inkjet deposition of bacteria are: (1) the size of cells and the aperture of the jet, (2) the bacterial solution fluid properties (e.g. viscosity) should match those of printer ink, and (3) the transfer method should maximise application of inducer or cells onto media. Variables assessed included paper type, cell concentrations, and pixel resolution.

An image was composed in Powerpoint software and then printed onto card stock paper, which was used to

Table 2: BioBrick parts constructed in this project

Name	Type	Description	Length
Bba_J21000	Composite	LuxR mCherry, TetR ECFP, LacI EYFP	2916
Bba_J21001	Reporter	LuxR controlled RFP	932
Bba_J21002	Regulatory	Promoter + LuxR	998
BBa_J21003	Regulatory	Promoter + TetR	904
BBa_J21004	Regulatory	Promoter + LacI	1372
BBa_J21005	Reporter	LuxR, TetR generator	2829
BBa_J21006	Regulatory	LuxR, TetR, LacI generator	1910
BBa_J21007	Regulatory	LuxR, TetR, LacI generator	3290
BBa_J21008	Composite	WaterColorDesign	6127

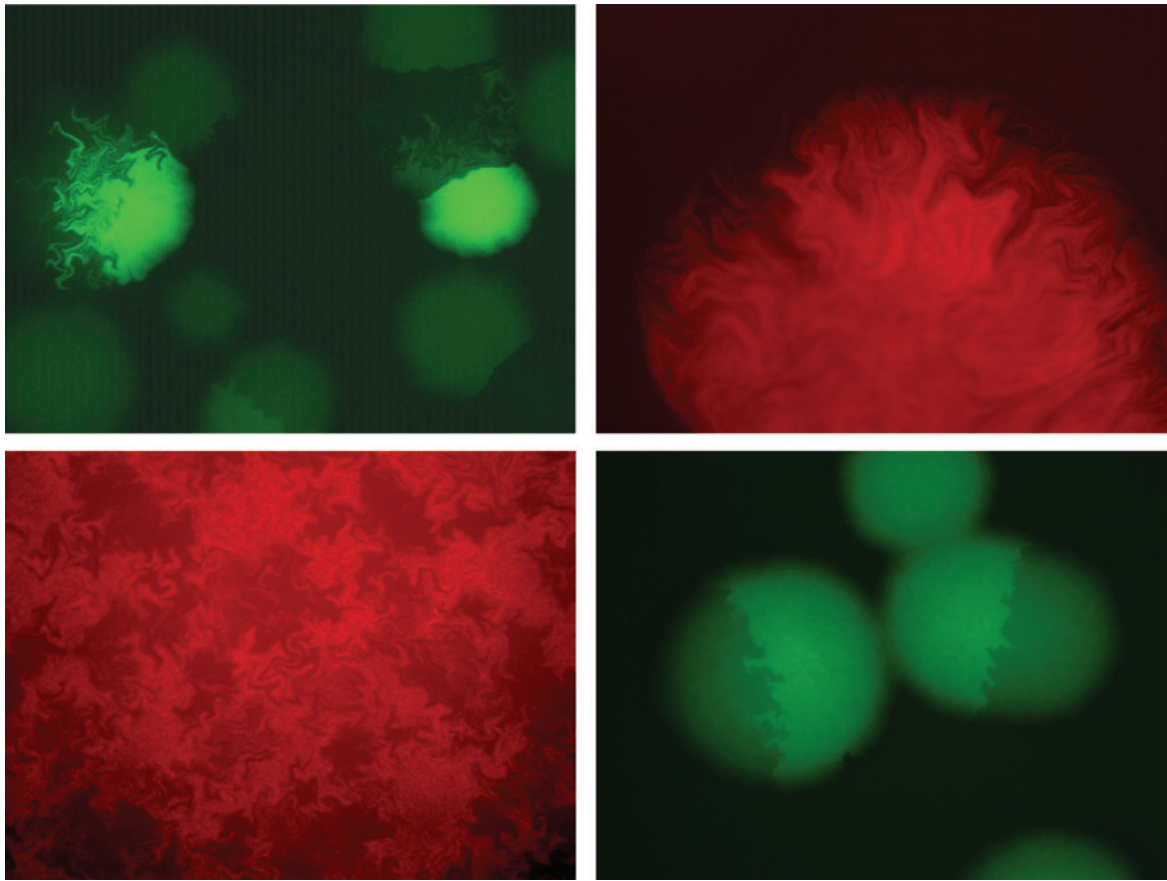


Fig. 2 *Images of fluorescent bacterial colonies*

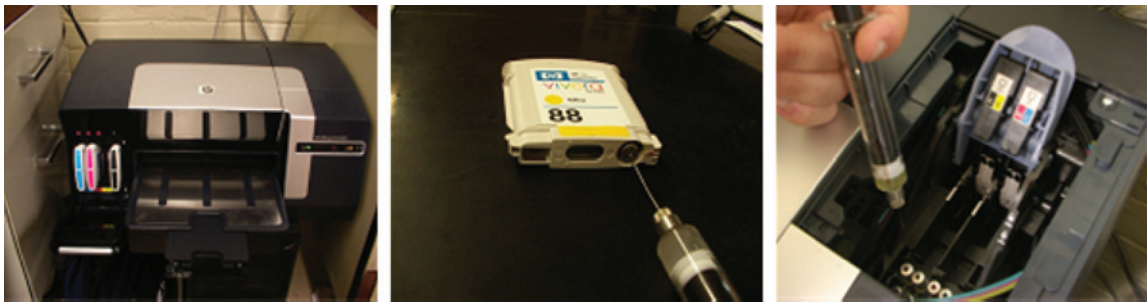


Fig. 3 *Loading bacteria into the inkjet printer*

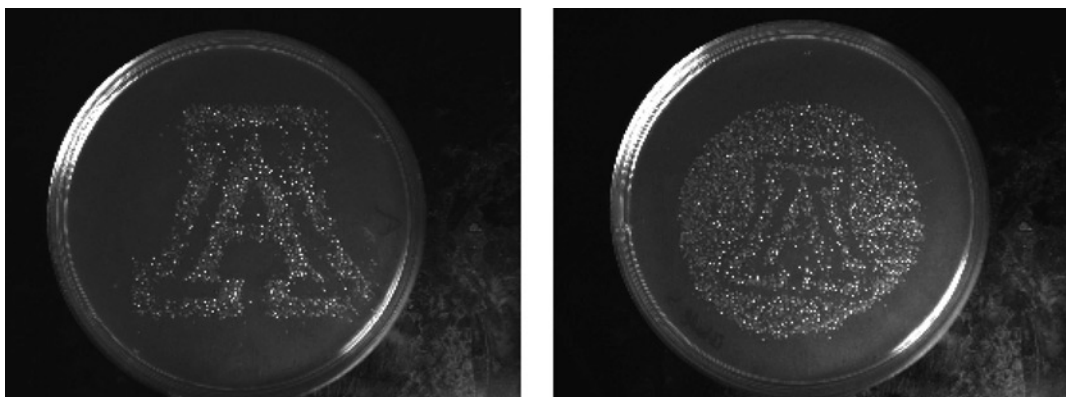


Fig. 4 *Images of bacteria delivered onto a Petri dish using a unique inkjet printing approach to form the UA logo*

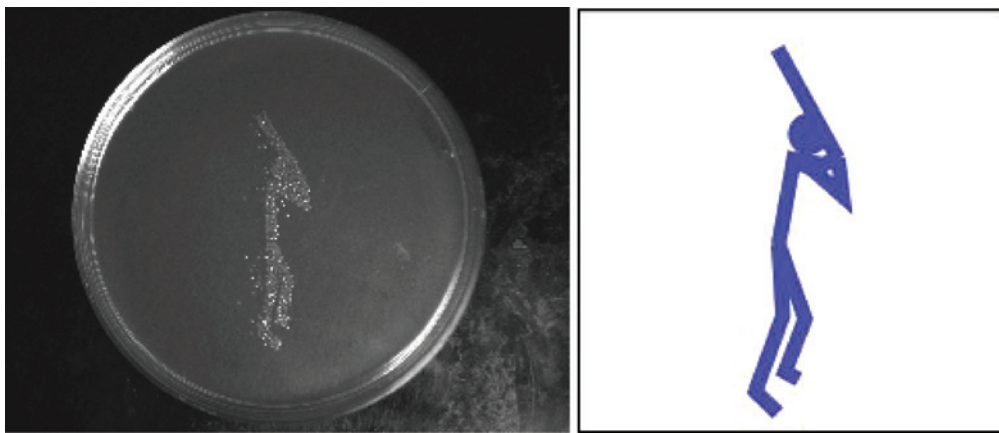


Fig. 5 Image comprised of bacteria of a baseball player

‘stamp’ a bacterial culture dish with the image. Images of printed bacteria are shown in Figs. 4 and 5. We could successfully print The University of Arizona ‘A’ logo and a number of images towards development of a bacterial movie of a stick figure playing baseball.

3 Conclusions

The conversion of a printer into a tool for distributing high resolution cell arrays was surprisingly straightforward and



Fig. 6 University of Arizona iGEM team members enjoying lunch at the 2006 iGEM competition at MIT's Stata Center (clockwise from left): Dr Joan Curry, Dan Reavis, Patrick Hollinger, Tyler Brown, Josh Kittleson, Kevin MacDow and Brian Heinze

is a practical tool that may be used in future projects. The registry of standard biological parts is an extremely useful resource which has allowed the creation of complex biological systems within a reasonable time frame. From a student's standpoint the project was a good opportunity to explore the field of bioengineering and to plan and execute a project. For future projects more emphasis will be placed on the planning and development stage as well as maintaining higher levels of organisation and communication.

This was a tremendous training experience for our team. Some members had previous experience working with DNA while others did not. The iGEM ambassador, Melissa Li, was a great resource especially in getting the project moving into the laboratory. She and University of Arizona technicians provided invaluable training. A team picture is shown in Fig. 6.

4 Acknowledgments

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Progress toward construction and modelling of a tri-stable toggle switch in *E. coli*

J. Lohmueller, N. Neretti, B. Hickey, A. Kaka, A. Gao, J. Lemon, V. Lattanzi, P. Goldstein, L.-K. Tam, M. Schmidt, A.S. Brodsky, K. Haberstroh, J. Morgan, T. Palmore, G. Wessel, A. Jaklenec, H. Urabe, J. Gagnon and J. Cumbers

Abstract: In 2000, Gardner and Collins reported the construction of a fundamental genetic regulatory device, the bi-stable toggle switch, in *E. coli*. We report here our work on a natural extension of this powerful device, a tri-stable genetic toggle switch capable of switching among three stable states. Like the bi-stable switch, the tri-stable switch consists of repressible promoters that produce inhibitory proteins and requires only a transient pulse of chemical inducer to switch among stable states. Our proof-of-principle construct is designed to control the expression of three different fluorescent reporters using the pBad/AraC, pLacI/LacI, and pTetR/TetR systems; though a tri-stable switch can theoretically be constructed from any three repressible promoters that satisfy a certain mathematical relationship. We have modelled the system extensively, creating both a simple continuous deterministic model based on the work of Gardner and Collins (Gardner and Collins, 2000) and a more complex discrete stochastic model based on the work of Isaacs (Isaacs, 2003). The tri-stable switch, designed, modelled, and partially constructed as an iGEM 2006 project at Brown University, is to be composed entirely of Biobricked parts from the Registry of Standard Biological Parts. In addition to providing support for the iGEM hypothesis, the tri-stable toggle switch has implications for biotechnology and gene therapy.

1 Introduction

Following construction of the first bi-stable toggle switch [1] much work has been devoted to the generation and study of bi-stable systems. The broad goals of this work have been to characterise bi-stable dynamics using mathematical modelling, to create bi-stable toggle switches from different genetic elements and in multiple organisms.

The bi-stable switch is a fundamental genetic element with great power and utility in a diverse set of contexts. Potential applications range from gene therapy to systems dealing with cellular memory. Additionally, to researchers they promise to be useful tools for transgenic experiments as they allow for the inducible switch between the stable expression of two transgenes when desired and without the need for prolonged exposure to inducer.

The tri-stable switch can function in each of these contexts while offering the additional control of another expression state. This additional expression state is non-trivial particularly when considered beyond our proof-of-principle design (Figs. 1a and 1b). For example, a system could be created in which there are two expression states and an ‘off state’. Alternatively, each state could be used to express the same protein at different levels creating a rudimentary transistor. The tri-stable switch could also be used as an element of a more complex system. It would be particularly interesting when employed in conjunction with

an oscillatory system which is built using an odd number of negative regulators. There are a variety of hypothetical payload configurations, implying the versatility of a tri-stable system. As yet unexplored, the dynamical properties of the tri-stable system are sure to differ from those of the traditional bi-stable system. Characterisation of these dynamics will lead to a deeper understanding of gene regulatory networks.

2 Aims of the tri-stable project

For Brown’s iGEM 2006 project we aimed to design, construct, model, and characterise a tri-stable toggle switch in *E. coli*. As our tri-stable switch was to be the first of its kind, we aimed to design a proof-of-principle switch that could be quickly constructed and easily characterised. We wanted to create a modular system – one that would require minimal modifications to be used in other contexts (i.e. to regulate the expression of other genes) by outside researchers upon obtaining it from the Registry. In order to provide mathematical validation for our initial intuition-based design logic and for future optimisation, we aimed to model the system extensively. Finally, we sought to characterise the tri-stable system and its parts. We hoped to fit the part characterisation data to the tri-stable model. Such an analysis would allow for the prediction of tri-stable system behaviour and ultimately lead to rational system optimisation.

3 Methods

Following design of the tri-stable switch, we began to model and construct the system concurrently. Fig. 2 summarises this process in more detail.

3.1 Design

Our proof-of-principal tri-stable switch was designed to control the expression of three fluorescent reporter genes: mCherry, ECFP, and EYFP, using the following promoter/repressor systems: pBad/AraC, pLacI/LacI, and pTetR/TetR, respectively. Fluorescent reporter genes were chosen as our GOIs (genes of interest) as they allow for simple quantification of gene expression. The promoter/repressor systems were chosen for their availability in the Registry and their thorough characterisation. See Fig. 1a and 1b for the conceptual and proof-of-principal designs of the tri-stable system. Analogous to the design of the bi-stable switch, each promoter expresses the repressors for each of the other two promoters and a fluorescent reporter (ex: pBad codes for LacI, TetR, and mCherry). Thus in the presence of one of the inducers, the system will express the fluorescent reporter gene and repress the other two promoters by expressing their corresponding repressor proteins. Theoretically, even after the removal of inducer, the expression state will remain stable as the active promoter will continue to generate proteins that repress the other two promoters.

The inducer-promoter pairs include the following: IPTG-pLacI, arabinose-pBad, aTc-pTetR. In order to create the most modular system possible, the system was designed to

be contributed to the Registry in three parts each containing only the promoters and repressor proteins. The genes to be expressed and their corresponding ribosome binding sites will need only to be added. Subsequently the constructs must be ligated together. Following characterisation of the complete construct and modelling, the system design will be further optimised by using RBSs of differing strengths and potentially using site directed mutagenesis. The final design element was the system chassis — what strain of *E. coli* to use. We eventually decided upon DH5 α as it does not produce LacI or TetR and only produces minimal amounts of AraC.

3.2 Construction

The construction of the tri-stable switch was divided among three groups of team members; each group was placed in charge of a separate inducer construct. To date, our lab work has resulted in the successful completion of the pBad construct in addition to various intermediate parts for the remaining two constructs. Each of the completed parts was sequenced and submitted to the Registry. A list of these parts can be found in the Registry [2]. Unfortunately, as alluded to above, numerous difficulties were encountered in the ligation procedure preventing the completion of the pLacI and pTetR constructs.

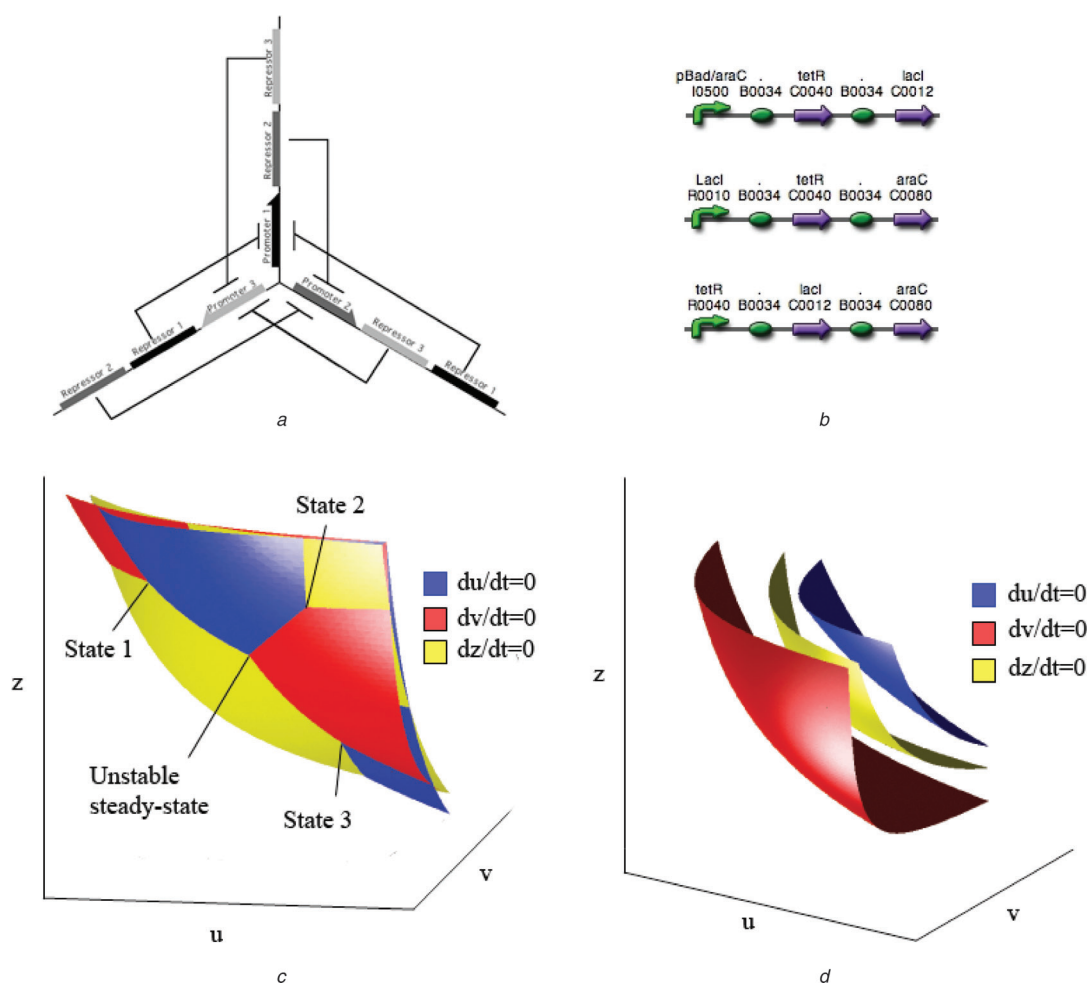


Fig. 1 Design and geometric structure of toggle equations

- a Conceptual design of the tri-stable toggle switch
- b Design of the three separate constructs of the tri-stable switch
- c A tri-stable toggle network with balanced promoter strengths
- d An unstable toggle network with unbalanced promoter strengths

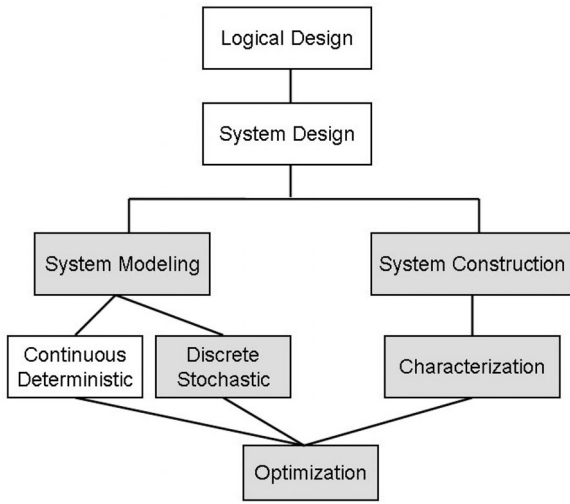


Fig. 2 Schematic of team goals; note that the grey denotes a work in progress

3.3 Modelling

In order to provide support for our initial intuition-guided design logic, we created a continuous deterministic model analogous to that in [1] comprised of the following three differential equations

$$\begin{aligned}\frac{dx}{dt} &= \frac{\alpha_{yx}}{1 + y^{n_{yx}}} + \frac{\alpha_{zx}}{1 + z^{n_{zx}}} - x \\ \frac{dy}{dt} &= \frac{\alpha_{yx}}{1 + x^{n_{yx}}} + \frac{\alpha_{zy}}{1 + z^{n_{zy}}} - y \\ \frac{dz}{dt} &= \frac{\alpha_{xz}}{1 + x^{n_{xz}}} + \frac{\alpha_{yz}}{1 + y^{n_{yz}}} - z\end{aligned}$$

The model supports the possibility of a tri-stable switch by verifying the existence of three stable expression states for a given set of model parameters. In this model u , v and z are protein concentrations and α and β denote effective synthesis rates of repressors and cooperativity, respectively (see [1] for a more rigorous description of the analogous bi-stable model). The three-way intersection points of the plotted null cline surface equations, $du/dt = 0$, $dv/dt = 0$, and $dz/dt = 0$, indicate steady states. Fig. 1c depicts a tri-stable system containing four such steady states (three that are stable and one that is unstable) while Fig. 1d displays an unstable system with no steady states. We are currently working to elucidate the set of parameters that lead to tristability for this model. In addition to this simple continuous deterministic model, we also sought to create a more complex discrete stochastic model that would allow for more accurate predictions of system behaviour and provide a source for more detailed optimisation. While not described here, our preliminary results from this model predict that with our current design, one promoter will dominate in the absence of inducer – leading to system instability (see the modelling section on the Brown iGEM team wiki for our progress on the discrete stochastic model [3]).

Characterisation of the system upon completion of the construct promises to yield more realistic model parameters and a trove of dynamic data. Other future goals include conducting a parameter scan on the discrete stochastic model to determine the set of parameters that will lead to tristability



a



b



c

Fig. 3 Brown iGEM team members in action

a Annie Gao carefully loads a gel to perform electrophoresis
b L to R: Victoria Lattanzi, Brendan Hickey, Azeem Kaka, Jason Lohmueller, and Megan Schmidt all hard at work in the lab
c Brendan Hickey attempts to extract DNA from Jason Lohmueller after the iGEM plates DNA has been exhausted

and performing system optimisation based the results of the system characterisation and parameter scan.

3.4 Characterisation

As only one of the three constructs has been completed there was little characterisation that could be performed. To date our characterisation has been comprised of a brief glimpse at DH5_s containing the pBad construct under the microscope. Their strong fluorescent red signal indicated that the endogenous expression of AraC had little to no effect on the expression of mCherry from the pBad promoter.

4 Conclusion

Despite having not yet completed the tri-stable toggle system, there have been many successful outcomes. First, we were able to successfully create the pBad construct, one of the three main system elements. Secondly, we were able to add this pBad construct and a variety of other intermediates to the Registry. Additionally, we have a set of core modelling equations and some interesting preliminary modelling results. Our continuous deterministic model predicts that a tri-stable switch is theoretically possible while our preliminary results from the discrete stochastic model suggests system instability. Following the completion of iGEM 2006, the Brown iGEM 2007 team has continued to work on the tri-stable toggle switch project. In the coming months, they hope to complete the remaining two constructs and begin the planned system characterisation. They also hope to further develop both models and optimise the system.

5 Brown iGEM 2006 team

The Brown iGEM 2006 team entered into the summer with much enthusiasm. As it was Brown's first year participating in iGEM, many of us had only just learned of synthetic biology and iGEM two months prior. While no one seemed to know exactly what to expect, there was a very real notion that this new scientific discipline and competition would endow us with both PI and even god-like powers; that we were to both design our own projects and engineer life.

Even as we continued to learn and the reality of what was possible and was not began to set in, to a great extent our grandiose expectations held true. Each project was our own and we were in fact engineering life. The nine undergraduate students on the team were given the freedom and power to invent project ideas and to play the central role in the development and construction of each project. Our faculty advisor and graduate students took on the roles of advisors and administrators, largely helping with issues in the lab and keeping the team organised. While as a team we are now considering increasing the level of faculty involvement for Brown iGEM 2007, the formula of an undergraduate-driven team worked quite well on the whole. It was an extremely rewarding experience for all of the students involved with only one major setback. As could be expected, many of our early ideas were overly ambitious and ultimately unrealistic. These ideas ranged from a cell division counter in *E. coli* to a treatment for cancer that involved targeting AMB-1 magnetosomes the nuclei of cancer cells. Unfortunately, our progress was hampered by ligation failures in addition to our late start.

Whether this was largely the result of simple lack of experience with iGEM or the undergraduate-run nature of the team is a subject of debate. Nonetheless, the summer was a veritable success. We emerged from iGEM 2006 having gained a unique and invaluable learning experience that will both bolster future Brown iGEM teams and our own research careers. Furthermore, we have set the foundations for two promising projects: the tri-stable toggle switch and the bacterial freeze tag. We intend to continue working on the tri-stable switch as part of iGEM 2007, and we are both excited and pleased to report our preliminary work on this project.

6 Acknowledgments

The system design and the experiments were performed by the team members were: Jason Lohmueller, Brendan Hickey, Azeem Kaka, Annie Gao, Jamie Lemon, Victoria Lattanzi, Peter Goldstein, Lick-Kong Tam and Meghan Schmidt. Faculty advisors were Alexander S. Brodsky, Karen Haberstroh, Jeffrey Morgan, Tayhas Palmore and Gary Wessel. Graduate mentors were Ana Jaklenec, Hayato Urabe, James Gagnon and John Cumbers. Jason Lohmueller and Nicola Neretti developed the model. Gary Wessel was the lead faculty and John Cumbers the main team organiser. The 2006 Brown iGEM team was funded by the Office of the Dean of the College, Office of the President, The Atlantic Philanthropies and the Departments of Molecular Biology, Cell Biology and Biochemistry, Molecular Pharmacology, Physiology and Biochemistry, the Division of Engineering and the Center for Computational Molecular Biology. The team was mentored by the Houseknecht lab at Pfizer, Groton, Connecticut. The project would not have been possible without the cross campus faculty support from: Arthur Salomon, Brian Moulton, David Rand, David Targan, James Valles, Jay Tang, Marc Tatar, Marjorie Thompson, Robert Hurt, Sorin Istrail, William J. Suggs, Thompson, Nancy, Herman Vandenburg, Vesna Mitrovic, Thomas Webster, Katherine Patenaude and the staff of the Brown Multidisciplinary Laboratory.

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New tools for self-organised pattern formation

K. Bernhardt, E.J. Carter, N.S. Chand, J. Lee, Y. Xu, X. Zhu, J.W. Ajioka, J.M. Goncalves, J. Haseloff, G. Micklem and D. Rowe

Abstract: Position-dependent gene expression is a critical aspect of the development and behaviour of multicellular organisms. It requires a complex series of interactions to occur between different cell types in addition to intracellular signalling cascades. We used *Escherichia coli* to study the properties of an artificial signalling system at the interface between two expanding cell populations. We genetically engineered one population to produce a diffusible acyl-homoserine lactone (AHL) signal, and another population to respond to it. Our experiments demonstrate how such a signal can be used to reproducibly generate simple visible patterns with high accuracy in swimming agar. The producing and responding cassettes of two such signalling systems can be linked to produce a symmetric interface for bidirectional communication that can be used to visualise molecular logic. Intracellular feedback between these two cassettes would then create a framework for self-organised patterning of higher complexity. Adapting the experiments of Basu *et al.* (Basu *et al.*, 2005) using cell motility, rather than a differential response to AHL concentrations as a way to define zones of response, we noted how the interaction of sender and receiver cell populations on a swimming plate could lead to complex pattern formation. Equipping highly motile strains such as *E. coli* MC1000 with AHL-mediated auto-inducing systems based on *Vibrio fischeri* luxI/luxR and *Pseudomonas aeruginosa* lasI/lasR cassettes would allow the amplification of a response to an AHL signal and its propagation. We designed and synthesised codon-optimised auto-inducing luxI/R and lasI/R cassettes as optimal gene expression is crucial for the generation of robust patterns. We still have to complete and test the entire genetic circuitry, although by modelling the system we were able to demonstrate its feasibility.

1 Aims

Our aim was to induce self-organised pattern formation in free swimming bacteria with techniques of genetic engineering. This was to be done by artificially introducing a system for bi-directional communication between two bacterial populations. *Escherichia coli* cells would be equipped with genes derived from independent quorum sensing systems from *Pseudomonas aeruginosa* and *Vibrio fischeri*. These systems can facilitate both communication between cell populations and regulated switching between competing cell fates. The negotiation of cell fates within bacterial populations can be visualised precisely by expression of different fluorescent proteins.

2 Proposed system

In our proposed system (Fig. 1), cell type 1 contains a lux sender cassette for the stable production of 3-oxohexanoyl

homoserine lactone (OHHL). The background expression of luxI from the P_{lux} promoter is sufficient for some OHHL to be synthesised by the luxI protein. This OHHL is then bound by the luxR protein, which is also expressed. Further activation of the P_{lux} promoter by OHHL-luxR complexes ensures that, over time, P_{lux} becomes locked in a fully active state by this positive feedback mechanism. As a consequence, OHHL is continuously produced and diffuses away as a signal molecule. The OHHL-receiver cassette of cell type 2 can thus be induced by such an OHHL signal to express green fluorescent protein (GFP). This activation is similar to the positive feedback seen in the OHHL-sender cassette. The rate of transcriptional activation from P_{lux} can be markedly increased by complexes of OHHL and luxR protein, which is also expressed in cell type 2.

At the same time, cell type 2 contains a las sender cassette for the auto-inductive production of a 3-oxododecanoyl homoserine lactone (ODHL) signal. The mechanism here is exactly analogous to the OHHL-sender cassette. ODHL can diffuse away from type 2 cells and induce transcriptional activation at the P_{las} promoter of the ODHL-receiver cassette in cell type 1. Consequently red fluorescent protein (RFP) is expressed. The crosstalk between opposite sender and receiver cassettes confers symmetric bi-directionality upon this signalling interface between both cell types.

3 Modelling

We built models for our proposed genetic system at both unicellular and multicellular levels. The single-cell model describes the transcription and translation activities inside a cell containing either type 1 or type 2 genetic circuitry. Assuming that both types of cells are similarly efficient in transcription and translation, the model characterises the

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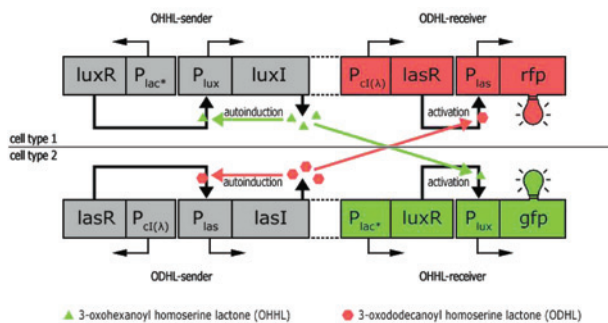


Fig. 1 System schematics. P_{lac^*} is a repressible hybrid promoter consisting of the -35 and -10 boxes from $P_{ci(\lambda)}$ and two $lacI$ repressor binding sites

rfp: red fluorescent protein
gfp: green fluorescent protein

system by showing stable auto-induction and switching behaviours and provides a theoretical basis for the feasibility of such a system. The multicellular model aims to simulate many cell–cell interactions on a swimming agar plate under the same assumptions as the unicellular model, and that both cell types are equally responsive to the two AHL signals, and that no cells die during the process. The simulation program shows that defined patterns can be generated via the movements of two types of cells and the diffusion of both AHL compounds. These simulated patterns furthermore agree with the real patterns that we observed in our experiments (Fig. 2a).

4 Experiments

We conducted a series of experiments to verify whether the proposed system would behave according to our predictions. In the first experiment we standardised the swimming assay for different strains of *E. coli* and relied on differential cell motility for pattern generation. We tested a number of *E. coli* strains for the swimming assay and found that MC1000 was the most motile strain with an estimated maximum rate of movement of 0.5 cm/h. In the second experiment we transformed different strains of *E. coli* with genetic circuits from the MIT Registry of Standard Biological Parts to induce expression of green, cyan, yellow and red fluorescent proteins. Experimentally it was easiest to distinguish green fluorescent strains from red fluorescent strains because green and red fluorescent proteins emit light of relatively high intensity in sufficiently different spectral regions. Using these fluorescent strains we were able to generate vivid patterns on a swimming plate

(Fig. 2). In the third experiment we equipped *E. coli* with biological parts to render them capable of AHL production. AHL synthesis and diffusion was confirmed by co-inoculation assays using *Chromobacterium violaceum* CVO26 [1]. In the last set of experiments we created two distinct populations of *E. coli* MC1000 cells by transforming them with different AHL sender and receiver cassettes and observed their behaviour in swimming agar. The interactions between these populations can generate defined patterns with high accuracy and remarkable sharpness of its edges and corners (Fig. 2b).

5 Synthesis of las and lux cassettes

We designed and synthesised codon-optimised las and lux sender cassettes (Fig. 3) that would allow optimal gene expression and the generation of robust patterns. It is important to note that the rate of auto-induction by both sender devices can be reduced by co-expression of a suitable repressor protein. The $P_{ci(\lambda)}$ promoter of the ODHL-sender cassette in cell type 2 is readily repressible by the CI repressor from phage λ . This promoter is characterised by a large range of activity between full activation and full repression. For maximum similarity, the P_{lac^*} promoter was chosen as an equivalent component in the OHHL-sender cassette of cell type 1. It is a hybrid promoter that has, in its unrepresed state, the same affinity for RNA polymerase as $P_{ci(\lambda)}$, and can be repressed to the same extent by the LacI protein instead of CI by virtue of two substituted repressor binding sites. Similarly, both sender cassettes also bear these two promoters such that the fluorescence response of each cell type can also be independently damped by co-expression of the respective repressor. The LacI and CI repressors can thus be used as the output of a separate molecular logic. If a secondary genetic circuit establishes characteristic concentrations of these repressors, it will be possible to use the fluorescence response as a readable output from this logic.

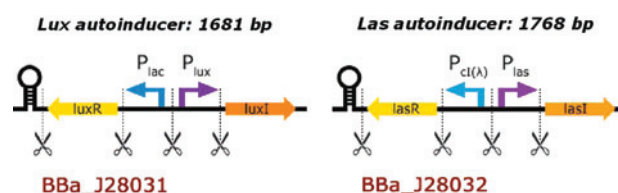


Fig. 3 Auto-inducing lux and las sender cassettes that were synthesised de novo

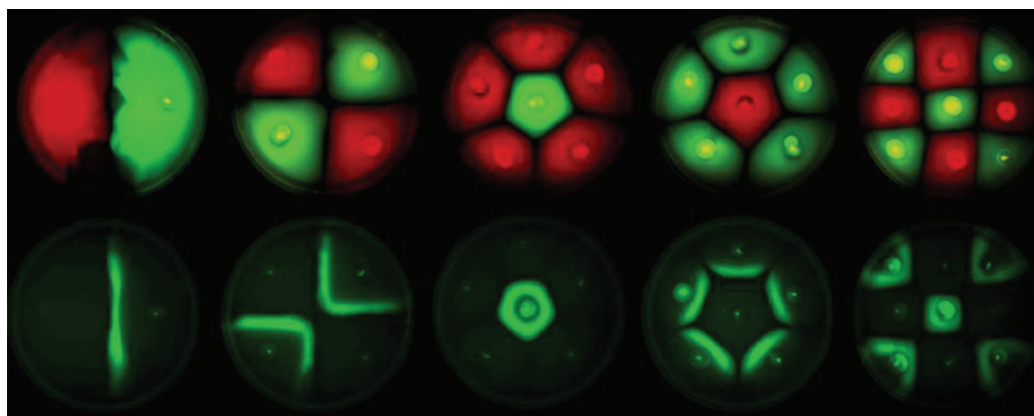


Fig. 2 Patterns on swimming agar plates

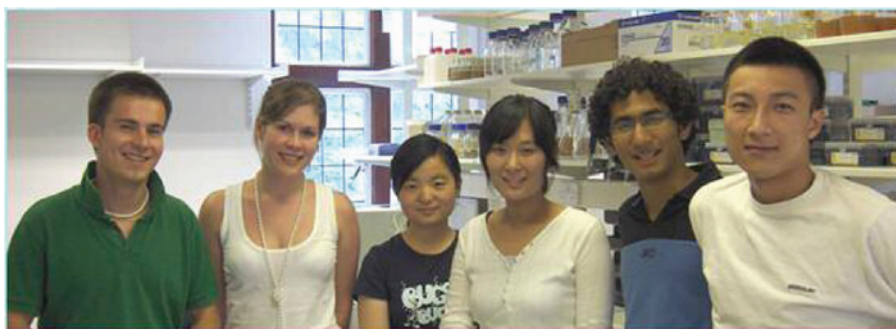


Fig. 4 *iGEM 2006 Cambridge team: Kaj Bernhardt, Elizabeth J. Carter, Xueni Zhu, Jisun Lee, Nikhilesh S. Chand and Yang Xu (left to right)*

Furthermore, the presence of unique restriction sites within the two sender cassettes will allow the replacement of any gene and promoter in the cassettes. We have submitted these cassettes to the MIT Registry of Standard Biological Parts with identifiers BBa_J28031 and BBa_J28032 (Fig. 3).

6 Conclusions

Our models identify that the proposed system has stable behaviour at the single-cell level and it is possible to generate defined zones of gene expression via cell movements and interactions. In practice the greatest variation was due to inaccurate inoculations and variations in the density of the swimming agar, which also affects the rate of movement. The fact that our experiments displayed patterns of higher precision than our multicellular models were able to generate is encouraging and may indicate that the bi-directional AHL-based signalling framework that we propose, in conjunction with differential cell motility, holds promise for future applications in directing position-dependent gene expression and forming patterns of higher complexity.

7 Acknowledgments

7.1 Teaching support

The students of the University of Cambridge team for iGEM2006 (see Fig. 4), and the supervisors, Dr Jim Ajioka, Dr Jorge Goncalves, Dr Jim Haseloff, Dr Gos Micklem and Dr Duncan Rowe, thank James Brown, Dr Jason Chin, Dr Gillian Fraser, Dr Keith Johnstone, Dr Matthew Levin, Dr Pentau Liu, Dr Jan Lowe, Dr Rita Monson, Dr Tony Southall and Dr Glenn Vinnicombe for their generous support during the competition.

7.2 Our sponsors

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Cell surface streptavidin

P. Tsai

Abstract: The goal of this project was to express streptavidin on the outer surface of *Escherichia coli* so that we could target any biotinylated nucleic acid or peptide to a cell surface. We used BioBrick assembly to fuse together a lipoprotein signal peptide, outer membrane protein A transmembrane domains and streptavidin protein domains. Assembly of constructs was completed and confirmed by PCR and sequencing. Protein expression was confirmed by western blotting with anti-streptavidin and anti-His6 antibodies. Protein functionality was confirmed by binding biotinylated and fluorescently tagged oligonucleotides to whole cells, but binding was also observed with non-biotinylated oligonucleotides.

1 Introduction

The problem of targeting materials to the surface of cells faces several hurdles. First, the identity of the material can make it tricky. DNA is not often found outside cells and so is neither naturally evolved nor easily engineerable (except in the presently limited research into aptamers) for recognising extracellular components. There are many endogenous secreted and membrane-bound proteins that bind to receptors, but it is not so easy to design targeting into the structure of a novel protein. Directing whole cells to other cells combines the problems of expression and membrane anchoring on two sides.

One potential system of cell surface targeting can be found in the well-characterised, strong non-covalent association between the protein streptavidin and the small molecule biotin. This association has been estimated with a dissociation constant of $\sim 10^{-15}$ M [1] and has been utilised heavily in laboratory purification techniques. Streptavidin is secreted by *Streptomyces avidinii* bacteria, and both its structure and sequence have been determined. Biotin, also known as vitamin H, is commonly attached to nucleic acids or proteins in a process called biotinylation. The goal of this project is to express streptavidin protein on the outer surface of *E. coli* so that biotinylated DNA or peptides can be generally targeted to the cell surface.

To express streptavidin on the surface, we created a fusion protein between the Lpp (lipoprotein) signal peptide, OmpA (outer membrane protein A) transmembrane domains, and streptavidin. This Lpp-OmpA surface display vehicle was developed by Francisco *et al.* [2], when they successfully expressed beta-lactamase on the outer surface of *E. coli*. A schematic is shown in Fig. 1a.

To fuse these protein domains together, we utilised a modified BioBrick assembly technique, developed by Silver and Phillips [3]. The spacer nucleotide immediately flanking BioBrick parts was not included so that the ligation site between sequences would have a length of six base pairs and the reading frame would be maintained.

Over the summer, BioBrick parts were fabricated for the fusion protein through PCR. The 'Lpp' part coded for the first 29 amino acids of lipoprotein, to target the fusion protein to the outer membrane. The 'O1' and 'O5' parts coded for one transmembrane domain (amino acids 46–66) and for five transmembrane domains (amino acids 46–159), to anchor the fusion protein in the outer membrane and direct the streptavidin outward. Both one and five transmembrane domains were used successfully by Francisco *et al.* The 'W' streptavidin clone codes for wild-type streptavidin monomer, while 'H' streptavidin clone codes for a wild-type streptavidin monomer plus a His6 tag at the C-terminus [4]. The 'S' streptavidin clone codes for a single-chain dimer streptavidin, also with a C-terminal His6 tag [5]. These parts were assembled by Phillips/Silver BioBricks assembly into the constructs listed in Table 1, and then the constructs were inserted downstream of a lac promoter and ribosome binding site (Fig. 1b) in pSB1A2 BioBricks vectors, which were transformed into Top10F' *E. coli* from Invitrogen.

In autumn 2006, work was done to confirm assembly of the constructs, to assess the expression of these constructs, and to test the functionality of the fusion protein to display streptavidin on the cell surface.

2 Results

To confirm that the constructs were successfully assembled, colony PCRs of the DNA constructs were performed and analysed by gel electrophoresis. The bands in Fig. 2 are at the expected sizes for each full construct. The constructs were later sequenced and confirmed.

To assess the expression of the fusion proteins, cell lysates from IPTG-induced cultures were run through an SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed by western blot using anti-streptavidin and anti-His6 antibodies, in Figs. 3a and 3b, respectively. Red bands (anti-streptavidin) appear at the expected sizes under all streptavidin constructs, and green bands (anti-His6) appear at the same bands under the streptavidin constructs which include a His6 tag ('H' and 'S'). There is a second band appearing above the band corresponding to construct size in the lanes from IPTG-induced LO1W and LO1H samples, which does not appear in the lanes non-IPTG-induced LO1W and LO1H samples. This difference between induced and non-induced samples was not

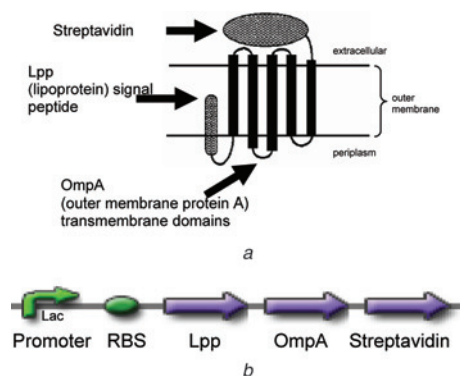


Fig. 1 *Lpp-OmpA fusion protein*

a Schematic of Lpp-OmpA-streptavidin fusion protein in outer membrane, with five transmembrane domains
b Gene map of fusion protein construct downstream of lac promoter and ribosome binding site

Table 1: List of construct names, parts, and approximate length of the construct in base pairs

Construct	Membrane part	Streptavidin part	Length (bp)
LO1	Lpp + OmpA, one TM	None	150
LO1W	Lpp + OmpA, one TM	Wild-type monomer	540
LO1H	Lpp + OmpA, one TM	Wild-type monomer plus His6 tag	560
LO1S	Lpp + OmpA, one TM	Single-chain dimer	970
LO5	Lpp + OmpA, five TMs	None	430
LO5W	Lpp + OmpA, five TMs	Wild-type monomer	820
LO5H	Lpp + OmpA, five TMs	Wild-type monomer plus His6 tag	840
LO5S	Lpp + OmpA, five TMs	Single-chain dimer	1250

observed in any of the other constructs, and no bands were observed from LO1 and LO5 samples (data not shown).

To test the functionality of the constructs, IPTG-induced cell cultures of the LO1 constructs were probed by on-cell western blotting with fluorescently tagged oligonucleotides. The whole cells were then pelleted, washed, and scanned for fluorescence. In Fig. 4a, cells which were incubated with no oligonucleotide showed no fluorescence. In Fig. 4c, cells were incubated with a fluorescently tagged streptavidin

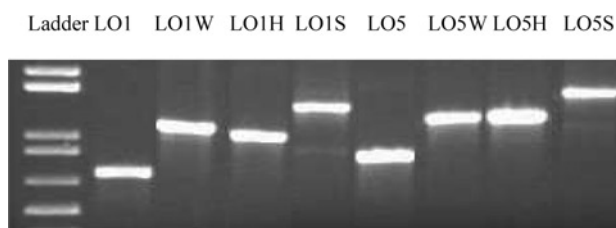
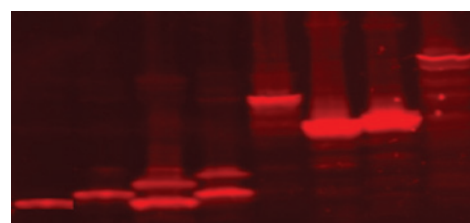
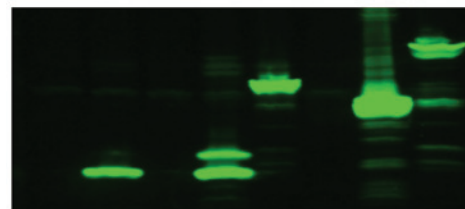


Fig. 2 *Agarose gel of colony PCRs*



LO1W LO1H LO1W LO1H LO1S LO5W LO5H LO5S

a



LO1W LO1H LO1W LO1H LO1S LO5W LO5H LO5S

b

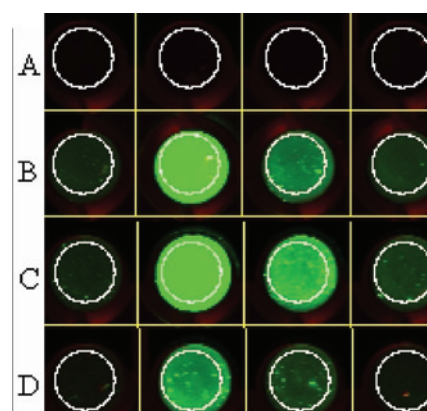
Fig. 3 *Western blot of cell lysates*

a Using anti-streptavidin

b Using anti-His6 antibodies

First two lanes in each scan represent non-IPTG-induced samples. All other lanes represent IPTG-induced samples

aptamer, a DNA oligonucleotide which should bind strongly to streptavidin protein. There was strong fluorescence on LO1W, medium on LO1H, weak/none on LO1S and on LO1. In Fig. 4d, cells were incubated with 'B-F' hybrid, a 5'-biotinylated oligonucleotide annealed to a 5'-fluorescently-tagged oligonucleotide, which acted like a single oligonucleotide with biotin on one end and fluorescent tag on the other. There was medium fluorescence on LO1W, weak on LO1H, and none on LO1S and on LO1. In Fig. 4b, cells were incubated with only the 'F' 5'-fluorescently-tagged oligonucleotide, which should have served as a negative control, as there is no biotin to bind to streptavidin. However, the results in Fig. 4b show fluorescence similar to the results in Fig. 4c.



LO1 LO1W LO1H LO1S

Fig. 4 *On-cell western blot*

a With no oligonucleotide

b With only 'F' 5'-fluorescently-tagged oligonucleotide

c With fluorescently tagged streptavidin aptamer

d With 'B-F' hybrid of 5'-biotinylated oligonucleotide annealed with 5'-fluorescently-tagged oligonucleotide

3 Discussion

The results of the colony PCR and sequencing verify that the full constructs have been assembled. The western blot shows that the fusion protein is being expressed, confirms that the assembled sequence is in-frame since the His6 tag is found at the end, and suggests that the streptavidin portion of the fusion protein can still fold in such a way that it is recognised by anti-streptavidin antibody. One point of interest is the extra band that appears in LO1W and LO1H under IPTG-induced conditions. Usually, bacterial proteins targeted for the membrane by a signal peptide (Lpp, in this case) have the signal peptide cleaved after membrane incorporation. What may be happening with LO1W(+) and LO1H(+) is that the small construct is being produced in such large quantities that not all of the protein is being incorporated into the membrane, and so some protein remains uncleaved and appears as this extra band corresponding to a slightly higher molecular weight.

The on-cell western gives more ambiguous results. There is a definitive positive result in that both the streptavidin aptamer and the B-F hybrid bound to the cells and produced greater fluorescence than the negative controls with no oligonucleotide and with LO1 construct which has no streptavidin portion. There seems to be weak or no fluorescence with the LO1S construct. This is unexpected considering that biotin should bind more strongly to a single-chain dimeric streptavidin because streptavidin occurs naturally as a tetramer. One possible explanation is that the dimeric streptavidin is not being expressed or displayed on the surface as efficiently as monomeric clones due to its sheer size, and less streptavidin on the surface would result in less biotin binding.

A puzzling result is the appearance of fluorescence in cells incubated with only the 'F' oligonucleotide. There is no 'B' biotinylated oligonucleotide annealed to it, and the sequence is randomised, so this should serve as a negative control which does not bind to surface streptavidin.

However, the results with the 'F' oligonucleotide look similar to the results with the streptavidin aptamer. This may represent nonspecific binding of the 'F' oligonucleotide, potentially to streptavidin on the cell surface, as no fluorescence appears on the cells with the LO1 construct that are without the streptavidin part. However, nonspecific binding of the 'F' oligonucleotide could imply non-specific binding of the streptavidin aptamer, thus somewhat weakening the implications of the fluorescence results with the aptamer.

It is also mysterious why there is a weaker signal from the LO1H sample than from the LO1W sample with all three assays, as the only difference between the two constructs is a histidine tag. The histidine tag may somehow be inhibiting oligonucleotide binding, or the histidine tag may be reducing the efficiency of surface expression of streptavidin. Another strange result is the generally weaker fluorescence in the 'B-F' assay in all samples. One possible explanation is that the single-stranded aptamer and 'F' oligonucleotide may bind to streptavidin better than double-stranded 'B-F' hybrid.

These data suggest that streptavidin is being expressed on the cell surface and binding to oligonucleotides, in comparison to cells without oligonucleotides or cells expressing constructs without streptavidin parts. However, it seems that oligonucleotides are binding nonspecifically, as the nonbiotinylated oligonucleotides produce even stronger fluorescence than the biotinylated hybrid.

These assays should be repeated with LO5 constructs in case those constructs produce better results. There should also be an assay comparing fluorescence results between single-stranded oligonucleotides and double-stranded hybrids, to attempt to explain the weaker fluorescence from the 'B-F' hybrid assay. It would be also be interesting to perform western blots on cell fractionated membrane samples to compare how much of the fusion protein is actually being incorporated in the outer membrane, which could explain differences in fluorescence results.

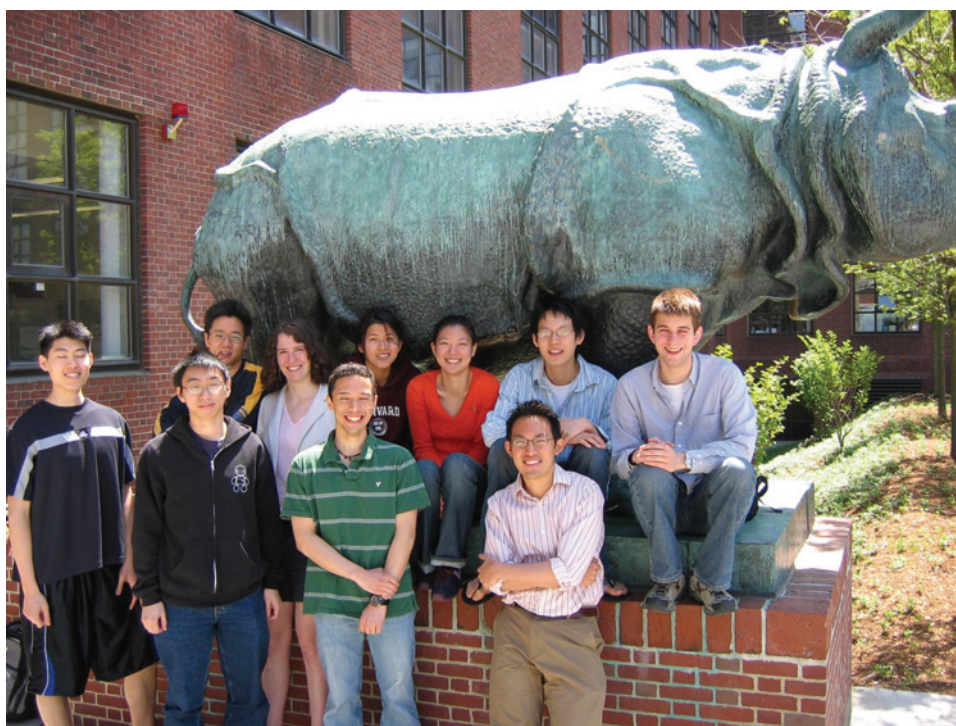


Fig. 5 *The 2006 Harvard iGEM team*

4 Acknowledgments

I would like to acknowledge Mark Howarth and Filiz Aslan for providing their streptavidin clones, Alain Viel for all his guidance and assistance in the laboratory, and the Harvard iGEM team (Fig. 5) and faculty for their support in motivating and initiating this project.

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Teaching bacteria how to dance

P. King, V. Lavrovsky, S. von Mammen and C. Jacob

Abstract: The motivational design idea for our iGEM 2006 team was to use bacteria to create swarm paintings. We designed two types of *E. coli* cells: senders and receivers. Stationary senders would act as attraction points; receivers would follow nutrition gradients towards the senders. In addition to the first steps of the actual *in vitro* designs, we developed a computer simulation of our bacterial swarm painters.

1 A great start, but then...

Since May 2006, the iGEM idea started to spark interest among students within the Faculties of Science and Medicine at the University of Calgary. Initially, ten students joined our iGEM team. Many aspiring projects were proposed, discussed and evaluated regarding their feasibility. Eventually, it turned out that most of our initial members had to leave the team as they were not able to commit on a full-time basis. Unfortunately, we had received our invitation to join the iGEM competition slightly too late to give our students enough opportunity to attain funding. This left three dedicated students to work on the actual implementation of the project (Fig. 1). So, what was the project idea?

2 Aims of our project

The key inspiration for our project came from SwarmArt.com [1], a collaboration between the Faculties of Art and Science at the University of Calgary, which has led to several interactive computer installations utilising swarm intelligence systems. Fig. 2 shows an interface through which an artist would create drawings through swarms. Painter agents, which leave trails of different colours behind, act like a simulated flock of birds (or bacteria?) which is attracted to a red dot. This target can be moved by the user and placed anywhere on the canvas. By moving the red target or leaving it at a location for a longer time, the swarms create interesting artistic compositions, in particular when obstacles are introduced into the scene. For our iGEM project, we wanted to recreate such an artistic swarm painter environment – with swarms of bacteria. Soon we realised that this endeavour is quite challenging as we wanted accurate control over a target, to have bacteria follow the target, and to produce

different colours (with fluorescent proteins) to reproduce the coloured trails in the computer model.

3 Bacterial painters

We decided to design two types of bacterial agents: senders and receivers. A Petri plate is inhabited by two strains of genetically engineered *E. coli* bacteria (Fig. 3). The first strain (the Senders) have been engineered to emit two chemical signals into the plate environment: aspartate and acyl homoserine lactone (AHSL). The senders themselves are activated by light (Fig. 4). This would allow us to simulate changing targets without having to wait for the sender bacteria to actually move. The second strain (the Receivers) have been designed to respond to each of these signals in a different way.

- The Receivers express green fluorescent protein in the vicinity of AHSL.
- The Receivers also move towards areas of greater aspartate concentration.

The same bacteria also decrease aspartate levels where they are present, as this is a nutrient and constitutes the reason for why they are attracted to it in the first place.

The goal was to utilise the Senders and Receivers to create interesting swarm drawings through bacterial interaction dynamics visualised by fluorescent patterns.

3.1 In silico model

In order to verify our sender-receiver system design, we implemented an *in silico* swarm-based model of interacting bacterial agents. We used the agent-based simulation environment NetLogo [2], which offers a powerful programming environment (with its own interpreted language), intuitive mechanisms to build user interfaces with sliders and buttons to control the simulation, and provides a fast visualisation engine. Fig. 5 shows the control panel and a two- and three-dimensional visualisation window.

Our bacterial sender and receiver agents are simulated within a virtual two-dimensional world. This discrete world consists of squares (so-called patches) over which the agents (so-called turtles) move. Patches can hold information, such as the concentration of AHSL and aspartate, which are visualised by different colours. The intensity of a colour reflects the actual concentration values; the higher a concentration, the darker is its representing colour patch. AHSL and aspartate are deposited onto



Fig. 1 Our iGEM 2006 Musketeers

Left to right: Sebastian von Mammen, Patrick King, Dr. Christian Jacob, and Vladislav Lavrovsky

patches by the sender agents. Subsequently, both AHL and aspartate undergoes diffusion across the patches.

A light source, which illuminates the whole simulation world, can be switched on and off by a button. As shown in Fig. 6, light-activated, immobile sender cells are represented in red. Motile receiver cells are originally black, but turn white, passing through green, proportional to how much AHL they detect on a patch. Once a receiver agent steps on a patch with a positive aspartate value, it consumes a certain amount (which can be set through a slider in the graphical user interface) of that patches aspartate.

Senders can be arranged in three different configurations: (a) along a small circle (Fig. 6), (b) in a cross-like fashion (Fig. 7), or (c) randomly distributed (Fig. 8). The visualisation window can display the senders and receivers, the AHL or aspartate concentrations.

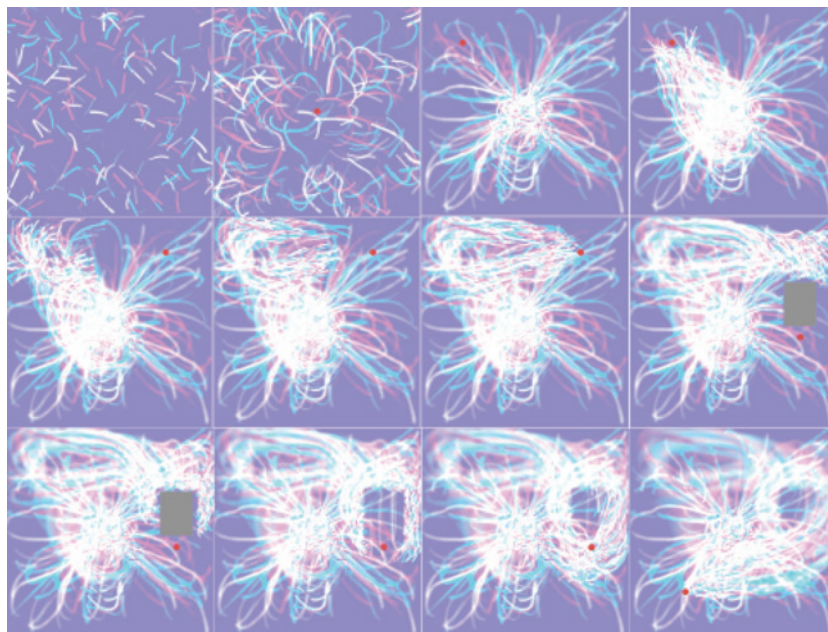


Fig. 2 Snapshots of a computer simulation of swarm painters

Painter agents are attracted towards the red dot, the position of which is controlled by a user. The painters leave trails of different colours behind, which eventually fade into the background. Obstacles, such as the grey rectangle, are engulfed by the swarms and reclaimed in case the object is removed

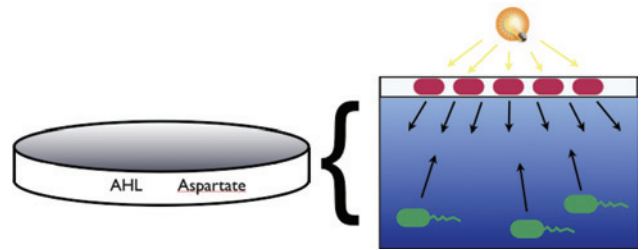


Fig. 3 Basic idea

Sender cells (in red) are activated by light. After activation, senders emit both AHL and aspartate. Receiver cells (in green) move towards higher aspartate concentrations, which attracts them to the senders. After detection of AHL, receivers express GFP

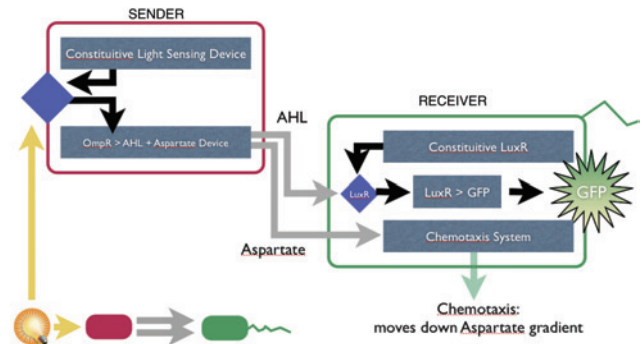


Fig. 4 Schematic of the design for the sender and receiver cells

3.2 The Wetware design

The final *in vitro* constructs were built using iGEM BioBricks from the MIT Registry, following our assembly plan in Fig. 9. PCR was used to produce workable quantities of the DNA as the part plasmids would often be low copy number and thus resulted in insufficient yields after plasmid purification. The BioBrick parts are contained on plasmids with ampicillin and/or kanamycin resistance

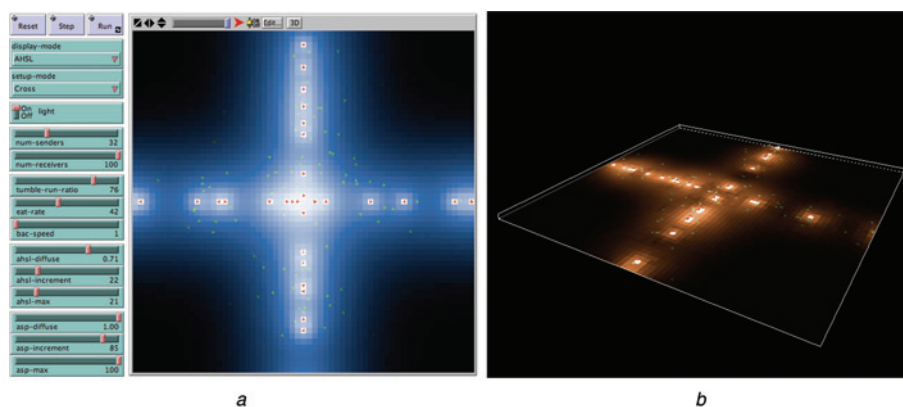


Fig. 5 Details of the user interface components for our *E. coli* simulator built in NetLogo

a Simple but effective interface to control parameter settings, as well as reset, start-stop, and step through the simulation
b 3D view of the simulation

markers and standardised upstream and downstream multiple cloning sites. We designed primers which are directly flanking these sites and thus work for any part in the registry. The primers are far enough outside the multiple cloning site to allow efficient digestion.

The typical construction went as follows. Two parts are selected and were amplified by PCR with Platinum Taq Polymerase (Invitrogen) using the generic primers. The part lengths were verified using 1% agarose electrophoresis

to ensure that the correct product was produced, they were also quantified using a NanoDrop spectrophotometer; parts were diluted to equal concentrations. The left part was then digested on the right flank with Spe I and the right part was digested with Xba I. Ligating the products together results in a permanent construct which has sites for neither of the restriction endonucleases Xba I and Spe I in between the two parts. This allows the addition of parts both upstream and downstream using the same method. In this way, part

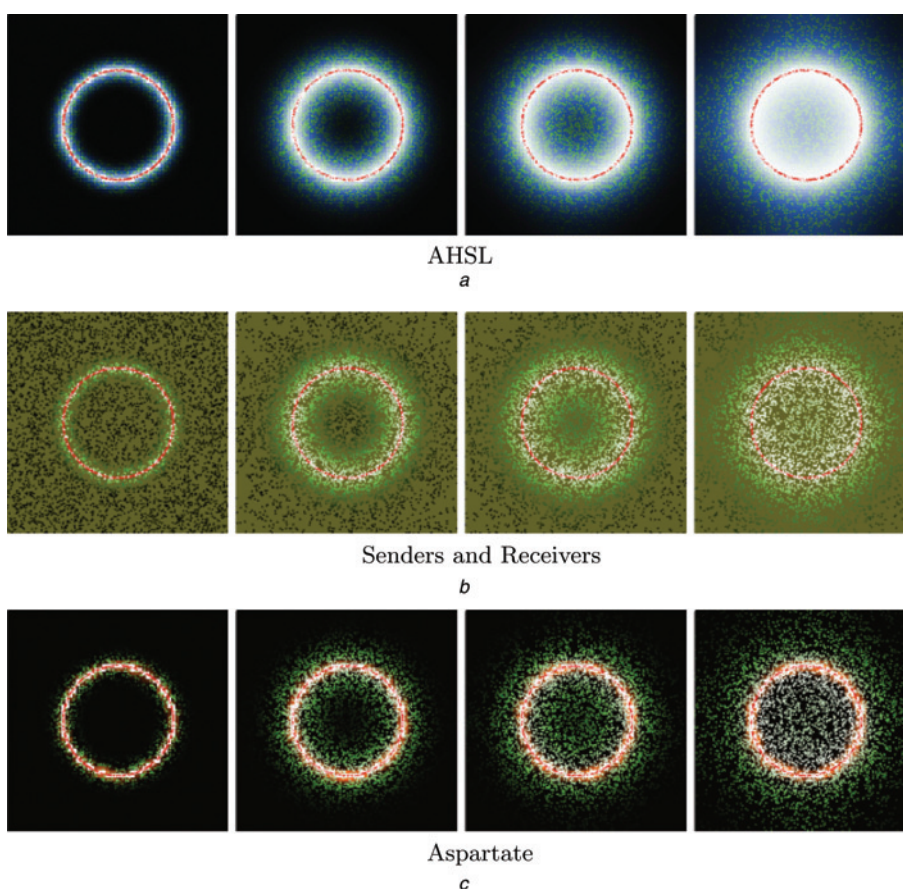


Fig. 6 Circular arrangement of sender cells

1000 sender cells, 5000 receiver cells

Snapshots are taken at the following iteration time steps: 0, 10, 22, 36, 46, 59, 74, 92, 117, 148, 187, 235, 295, and 370

a AHSL

b Senders and receivers

c Aspartate

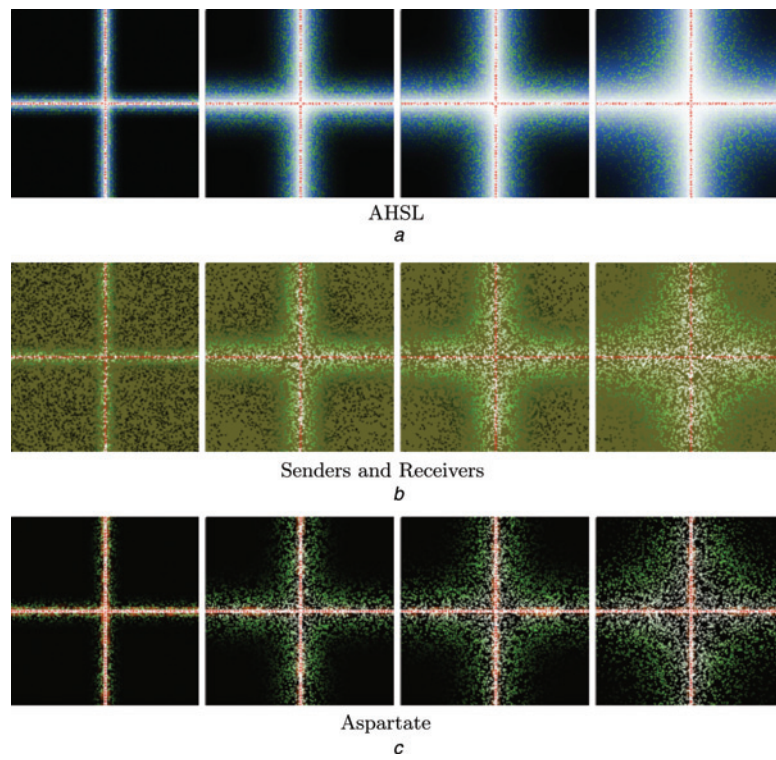


Fig. 7 *Cross arrangement of sender cells*

1000 sender cells, 5000 receiver cells

Snapshots are taken at the following iteration time steps: 0, 10, 22, 36, 46, 59, 74, 92, 117, 148, 187, 235, 295, and 370

a AHSL

b Senders and receivers

c Aspartate

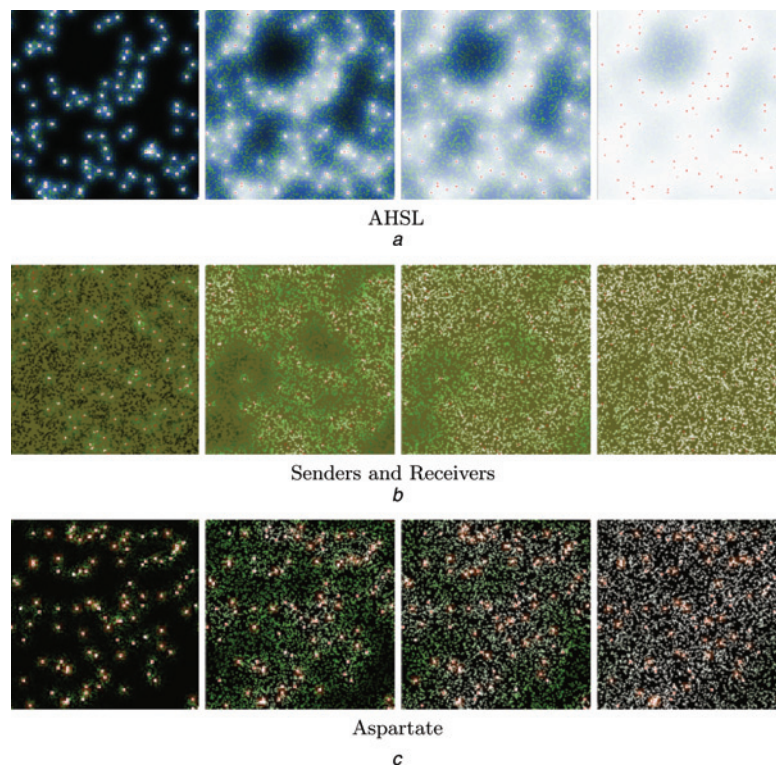


Fig. 8 *Random arrangement of sender cells*

Number of sender cells was reduced to 100

5000 receiver cells

Snapshots are taken at the following iteration time steps: 0, 10, 22, 36, 46, 59, 74, 92, 117, 148, 187, 235, 295, and 370

a AHSL

b Senders and receivers

c Aspartate

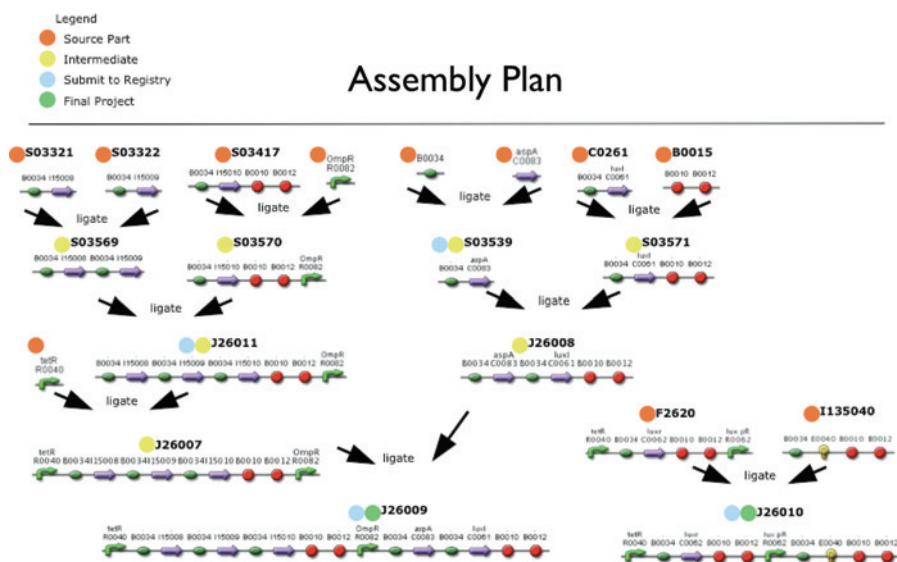


Fig. 9 Plan for the assembly of parts necessary to implement the senders and receivers

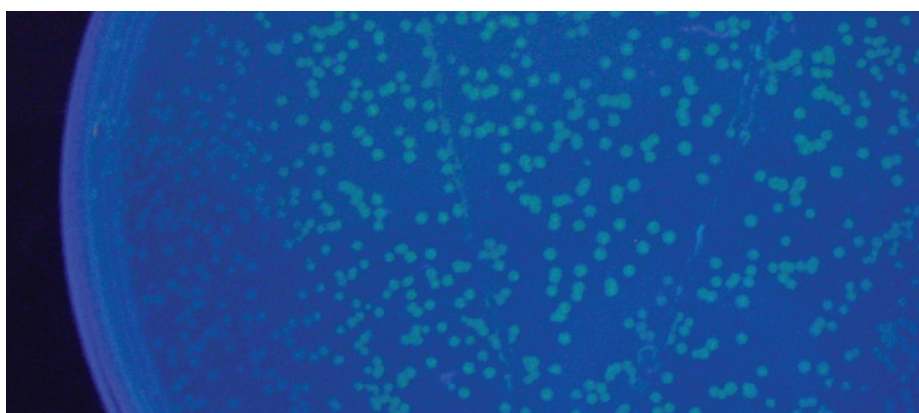


Fig. 10 AHL gradient

of the intended device was constructed. Part F2620, a constitutively expressed luxR gene, and a lux promoter was combined with part I13504, which is a GFP.

Thus, whenever AHL is present, GFP is expressed. In Fig. 10 this is demonstrated by adding a small quantity of AHL (Sigma) in the middle of the plate. As the AHL diffuses, GFP expression is induced.

4 Lessons learnt ...

We lost a bit of our momentum through the summer, especially when it came down to actually applying the creative designs in the wet lab. Three of us, however, kept the project afloat and were dedicated to bring it to a successful end – although this proved to be much harder than anticipated due to the time-consuming and error-prone nature of wet lab experiments. But this is part of the lessons to be learnt. Dealing with reprogrammed bacteria is not precision engineering, nor is it as straightforward as computer programming. We were quite anxious when it came to present our project at the iGEM Jamboree. To our great surprise, our team won the First Place in the Conquest of Adversity category, which recognised our team's effort to bring this project to a successful end. The current stage provides the first step for an expansion of the Dancing Swarm Bacteria for 2007.

5 Conclusion

The work done has set the stage for further work in the field. The use of PCR has so far been successful but there is still concern that many rounds of amplification can introduce mutations. Thus the method will be validated by serial amplification of a large part (2000 bp) for as many cycles as would be required to make the proposed construct. If after 30 cycles there are few or no mutations, it would be safe to continue to use PCR instead of isolating plasmid DNA. In the future we intend to add novel parts as well as devices into the registry, as well as build on our first-year experience to build more sophisticated genetically engineered machines.

6 Acknowledgments

We would like to thank Phillipa Sessini and Boris Shabash for their contributions to our project. We particularly thank Lisa Allen for supporting us in the lab.

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Escherichia coli robots that freeze, smell, swell, and time-keep

Y. Tashiro, M. Furubayashi, T. Morijiri, K. Suzuki, K. Yasuno, S. Matsuno, A. Katabami, K. Saito and D. Umeno

Abstract: As an extracurricular activity, 18 students in chemistry major tried to create four *Escherichia coli* ‘robots’: (1) an imaging system with *swimmy* bacteria; (2) a switchable aroma generator; (3) a balloon bacteria with a light-triggered inflator; and (4) an *E. coli* clock. None of the projects were completed, but a number of BioBricks were generated. They include an aroma synthesiser, a motility controller, and a size and shape controller. By assembling and tuning these BioBricks, we will be able to complete our robot manufacture. We believe these BioBricks would expand the toolbox in bacterial robotics.

1 Foundation of iGEM Chiba

Eighteen students in chemistry department got together to found the Chiba iGEM team. Our iGEM started off as the departmental extracurricular course to learn biology by firsthand experience. Another aim of iGEM Chiba was to quest for a chemists’ way to contribute to the iGEM society.

To maximise our source of learning, we intended not to define our project too early. From the kick-off (April 22 2006) to the beginning of August, each of us held his or her own project. Twice a week, we got together and helped each other to deepen each of our projects. In parallel, we filled the gap between the last biology classes we have taken (at junior high) and the minimum biology knowledge required for iGEM participants. Finally, we narrowed down the number of the projects. The four projects survived our version of democratic process (where the loudest voice rules). This process was later awarded a prize for ‘Most Creative Brainstorming.’

2 *Swimmy* bacteria

Inspired by the storybook *Swimmy* by Leo Lionni, we aimed to generate images by controlling the spatial density of bacteria. To fulfil this, we tried to engineer *E. coli* that swims in the dark but temporarily freezes its motility in the light. In the dark, the *E. coli* ‘robots’ swim in random directions. When they happen to enter the illuminated zone, they instantly get trapped. Over time, bacterial density of the illuminated zone becomes higher than the rest, revealing the image reflecting the shape of photo-mask. Because *E. coli* gets liberated from the paralysis by turning the light off, one can regenerate the images repeatedly by changing the masks.

Instead of importing the entire phototaxis system from other organisms, we tried to make our own by harnessing light sensor [1] with brakes and accelerators (Fig. 1a). In search for the controller of *E. coli* swimming velocity, we

found a paper on the function of mutants of chemotaxis receptor Tsr [2]. In the paper, various Tsr mutants were shown to cause excessive clockwise rotation of *E. coli* flagella motors (keeping *E. coli* in ‘tumble’ mode), while other Tsr mutants force flagella to rotate counter-clockwise (‘run’ mode) [2]. We thought that controlled expression of these mutants could provide a good way to brake or accelerate the motility of *E. coli*.

We PCR-cloned the Tsr_{CW} (clockwise mutant in pPA90 in [2]) and placed both under the arabinose and OmpC promoters. Under the microscope, *E. coli* showed a significant decrease in motility upon induction of Tsr_{CW}. On soft agar, however, Tsr_{CW} slowed down the swarming of *E. coli* colonies only by approximately 20%. Clearly, we needed a better *E. coli* trapper to generate sharp macroscopic images. We are presently listing all other mechanisms that could better control the swimming velocity of *E. coli*.

3 Aroma bacteria

We aimed to construct an aroma generator. Aroma fragrances possess healing capacity, and could be useful as a new class of output signal for bacterial sensors. Our nose distinguishes >10 000 different scents in high sensitivity. By harnessing the sensory units with scent generators, we can create whole cell sensors suitable for operation outside the limits of our sensory capacity.

Among various types of aroma molecules, we chose terpenes, the condensation product of isopentenyl diphosphates (IDP) available in all living organisms. They cover a wide range of scent and flavor, and terpenoid pathways are highly evolvable [3]. In the laboratory, one can mix, tune and even invent a wider set of aroma fragrances.

We have collected many aroma synthases and expressed them in *E. coli* in various conditions. Considering the real world application, we scored their performance using our own nose. Among them, pinene synthase and geraniol synthase generated most recognisable fragrance. Next, we systematically co-expressed these aroma synthases with various upstream enzymes that potentially elevate the production level of aroma. In our system, the biggest booster effect was resulted by the single addition of *E. coli* isopentenyldiphosphate isomerase (Idi).

Fig. 1b shows the overall construct we were to assemble. For light input, we planned to use Levskaya’s light sensor [1].

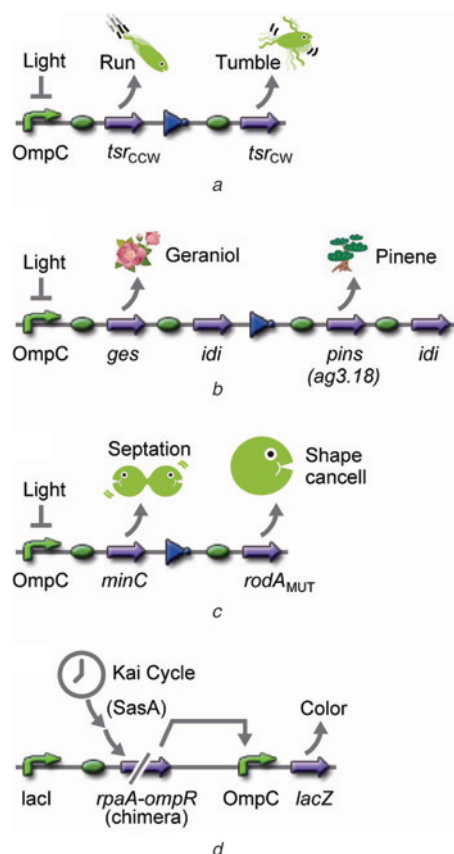


Fig. 1 Four projects conducted as iGEM CHIBA 2006

a Light-directed controller of the flagellar rotation
 b Switchable aroma generators
 c Light-induced cell inflator
 d Device to readout the Kai oscillation

In the light, *E. coli* harbouring this circuit generates pinene (refreshing aroma of pine tree). After dark, the fragrance is switched to that of geraniol (moody smell of rose).

Although we put all the components together, we could not test the light-directed switching of the aroma production. Luckily, the UT Austin team had generated a single plasmid light sensor in the course of their iGEM project. Using the plasmid, we believe we can complete this project.



Fig. 2 Chiba iGEMers 2006

Front row (left to right), S. Fukushima, M. Muramatsu, M. Ikezumi, K. Yasuno; second row, S. Sugaya, S. Okuyama, K. Megumi, K. Nakajima, D. Abe, K. Suzuki; back row, T. Kaneda, N. Kakimi, A. Katabami, S. Matsuno, M. Mihashi, Y. Asaari, T. Morijiri, T. Karasawa, Y. Tashiro

4 Balloon bacteria

Size and shape have much influence on cellular motility and logistics, and all cells have unique sizes and shapes appropriate for their natural lifestyle. However, it does not necessarily mean that these size and shapes are suitable for the job we order them to conduct. We investigated the possibility to control the size of *E. coli*.

There are many genetic factors that affect the size and shape of *E. coli*. For example, deletion of MinC protein (the protein playing a role in septum formation) results in noodle-like cells reaching to 20–50 μm in the long axis [4]. We reasoned that the size of cell in the minor axis remains the same owing to the proteins that make a robust cell liner. Some *rodA* mutants are reported to confer *E. coli* spherical shape [5]. We decided to combine the *rodA* mutations (shape canceller) with *minC* genotype (defect in septum synthesis) in the hope of making an *E. coli* inflator. In our original design (Fig. 1c), the inflator was under the control of light sensor [1].

From genomic DNA of *E. coli*, we PCR cloned *rodA* gene in mutagenic conditions. The resulting *rodA* library was placed under the lac promoter and transformed into DH10B. Among 48 variants screened with microscope, we found three mutants that impart a spherical shape to *E. coli*.

The RodA mutants above obtained were transformed into *minC* strain (JW1165 in KEIO collections [6]). Upon expression of the RodA mutants, JW1165 drastically altered its shape from noodle-like to spherical. However, the size of the cell became even smaller than the original strain (BW25113). Our current interpretation of this result is as follows. The loss of the defined structure in cell wall lining have either accelerated the cell division or slowed the rate of cell wall synthesis. Currently, we are studying the effect of kinetic balance between cell wall synthesis and septum formation on the overall size and shape of *E. coli*.

5 *E. coli* clock

The installation of a 24 h clock allowed us to make *E. coli* punctually do their work at a given time of the day. Unlike the other circadian clocks in nature, Kai system in cyanobacteria requires only three components (Kai A, B, and C) to generate the pulse, and the oscillation cycle looks almost temperature-independent [7]. Many researchers including the Harvard iGEM team, are trying to establish Kai oscillation in *E. coli*, but there reported no engineering effort to bridging between the Kai oscillation and gene expression.

We aimed to make a device that activates *E. coli* promoter in response to the Kai cycle (Fig. 1d). In the Kai system, the oscillation occurs in the phosphorylation level of KaiC proteins. Phosphorylated KaiC accelerates the phosphate transfer from SasA (EnvZ homolog) to RpaA (OmpR homolog) [8].

We systematically constructed chimeras between N-terminal 'receiver' domains of RpaA and C-terminal 'effector' domains of OmpR with 36 different crossover points. Functional chimeras should selectively receive phosphate from SasA and activate Omp promoters in KaiA, KaiC and SasA background, but not in KaiB, KaiC and SasA background.

We have not tested whether they function as Kai-independent Omp activators. But we recently found a paper on a chimera between OmpR and PhoB, another OmpR homolog [9]. In this paper, the authors argued that

OmpR requires its receiver domain to correctly interact with (and recruit) RNA polymerase; chimeras lacking these domains fail to activate Omp promoters. It is very likely that this applies to our RpaA-OmpR chimeras. Probably, we should have rather evolved receiver domain of the OmpR to selectively receive phosphates from SasA.

6 Conclusions

One painful lesson along these projects was that biological function is indeed context-dependent. For example, we could see the function of the light sensor [1] exactly in the recommended condition, but we kept failing either to make it function in other strains or to modify it for our own purposes. To make bacterial robotics efficient (and more fun), we need to pin down where we can plug and play BioBricks without unexpected outcomes. Alternatively, we should establish the quick and systematic procedure to tune any given circuits [10].

None of our robots are functioning as of today, but we were able to generate various types of output devices in BioBrick format. Also, we ourselves developed much skill and experience on bacterial robotics. In a little bit better shape, Chiba will be definitely back for iGEM2007!

7 Acknowledgments

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Computing with living hardware

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Abstract: Our multi-institutional team of eleven undergraduates, one high school student, one postdoctoral fellow, and four faculty members explored the emerging field of synthetic biology and presented our results at the 2006 international Genetically Engineered Machine (iGEM) competition. Having had little or no previous research experience, biology, chemistry and mathematics students from four different institutions collaborated during the summer and fall semester of 2006. We identified the burnt pancake problem (sorting by reversals) as a mathematical puzzle ideal for solving with 'living computer hardware': *Escherichia coli* cells programmed to sort tandem fragments of DNA by reversals (DNA inversions or 'flipping'). Flipping is driven by a *Salmonella typhimurium* Hin/hix recombinase system that we reconstituted as a collection of BioBrick-compatible interchangeable parts. We tested functionality of these synthesised genetic parts and mathematically modeled the behaviour of pancake flipping. The living hardware system allowed us to consider future research applications such as regulating genetic element rearrangements *in vivo* and DNA computing. We found the field of synthetic biology to be ideal for learning, teaching, sharing, collaborating, and conducting integrative and original research with undergraduates.

1 Aims of the project: a biological approach to solving a mathematical puzzle

Our team set out to engineer bacteria in order to build living computer hardware that can compute solutions to a mathematical puzzle called the burnt pancake problem. The puzzle can be thought of as a stack of different sized pancakes, each having one burnt side and one golden side, arranged in an arbitrary order. The goal is to rearrange the pancakes by flipping individual pancakes or subsets of adjacent pancakes until the pancakes are sorted from largest to smallest with each pancake facing golden side up. In computer science this process is called sorting by reversals. As the pancake stack becomes larger, the number of possible arrangements increases and the problem becomes computationally intractable. To produce essentially unlimited computing power, we decided to harness the power of *Escherichia coli* DNA replication and cell division. We

modified the *Salmonella* Hin/hix DNA recombinase system to perform DNA reversals on plasmid processors in a massively parallel processor (>200 plasmids per cell in a population of $\sim 1 \times 10^8$ cells/mL). Our system, affectionately called the *E. coli* House Of Pancakes (E.HOP) computer, is a proof of concept for computing *in vivo*. Mathematical modelling of random reversals helped us design the system and interpret the output of our E.HOP computer.

2 Description of the work

2.1 Building the E.HOP computer

The burnt pancake problem is ideal for demonstrating the computational capabilities of living hardware. The biological equivalent to a burnt pancake is a functional unit of DNA such as a promoter or coding region. Similar to burnt pancakes, expressed DNA elements have directionality (5' to 3'), require a specific order of the units (e.g., promoter followed by coding region) and can be flipped (cut, inverted, and spliced *in vivo* by cellular machinery). To flip units of DNA, we have reconstituted the Hin/hix recombinase system from *S. typhimurium* as a BioBrick-compatible set of components for use in *E. coli*. In *S. typhimurium*, native Hin recombinase activity is required for the inversion of a ~ 1 kb chromosomal segment that mediates the expression of the H1 and H2 flagellin genes during phase variation [1].

Hin recombinase was cloned from the *S. typhimurium* (Ames strain TA100) genome and tagged with the LVA degradation signal (part BBa_M0040 [2]) using PCR amplification. Hin-mediated DNA inversion requires the recombinational enhancer (RE), a cis-acting DNA element, and hixC, a symmetrical 26 bp sequence that is recognised by the Hin homodimer [3]. To construct these parts, we used the publicly available genomic sequence of *S. typhimurium* and a double-stranded DNA (dsDNA) assembly program we created for gene synthesis from overlapping oligos [4].

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Any segment of DNA capable of being inverted (i.e., a DNA pancake) must be flanked by a pair of *hixC* sites. Hin recombinase recognises these *hixC* sites and cleaves both strands of DNA. With the help of the Fis protein bound to the RE, the invertosome complex inverts the *hixC*-flanked DNA fragment [5, 6]. In our system, selectable phenotypes of antibiotic resistance depend upon the proper arrangement of a series of *hixC*-flanked DNA segments in a plasmid. A selectable marker allows us to detect which cells have successfully solved the puzzle.

Using several modes of communication (Fig. 1), our iGEM teams at Missouri Western State University (MWSU) and Davidson College cooperatively addressed specific design considerations. We selected segments of DNA that could be sorted to yield a phenotypically unique solution and we built several construct intermediates to optimise positioning of the *hixC* sites. Our final design is a two-pancake stack (Fig. 1); pancake 1 is a pBAD promoter (part BBa_I13453 [2]) from the arabinose operon and pancake 2 is a tetracycline resistance coding region with an upstream ribosomal binding site (RBS-Tet^R, part BBa_S03562 [2]). We designed an insulated vector called pSB1A7 that effectively prevents read-through from the vector backbone into the pancake stack [2]. An additional plasmid encodes the AraC and Hin invertase proteins. This AraC/Hin generator (part BBa_J3108 [2]) was designed to express Hin-LVA in the presence of IPTG and pause transcription (via AraC binding to pBAD) during Hin-mediated DNA inversion. After co-transformed cells have undergone random flipping, samples are grown in the presence of tetracycline to obtain colonies that carry a correctly sorted pancake stack. Before running the living hardware system, we mathematically modelled its behaviour to help us interpret the results.

2.2 Mathematical modelling of pancake sorting

Our mathematical representation of a burnt pancake stack is a signed permutation, in which each integer represents the pancake size and the sign of the integer represents the orientation. The permutation $(1, 2, \dots, n)$ denotes a sorted stack of n pancakes in order from smallest to largest, all golden side up. A negative sign denotes a pancake facing burnt side up. Note that for n burnt pancakes, there are $(2^n \times n!)$ possible arrangements. To visualise how many flips are required to sort each arrangement, we generated graphs in which the signed permutations comprise the vertices and a flip of a single or multiple adjacent pancakes is represented by an edge connecting two vertices (Fig. 2).

In these graphs, forward and reverse orientations of pancake stacks (i.e., $(1, 2)$ vs. $(-2, -1)$) are considered distinct, whereas in a biological system they are functionally equivalent. Cells carrying either (*pBAD*, *RBS-Tet^R*) transcribed from left to right or (*RBS-Tet^R* reverse, *pBAD* reverse) transcribed from right to left will express tetracycline resistance. Since both of these arrangements can be thought of as correctly sorted stacks, the modelling would have to be modified to determine the number of flips necessary to reach either configuration and only half of the signed permutations could be considered. To discriminate between the two biologically equivalent correctly sorted stacks, the DNA construct includes a stationary promoterless red fluorescent protein reporter (*RBS-RFP* reverse, part BBa_J31011 [2]) that is expressed when *pBAD* is in the reverse orientation (Fig. 1).

Some additional biological factors must be considered in order to model the behaviour of the living hardware system. Flipping might be biased for DNA fragment size, proximity

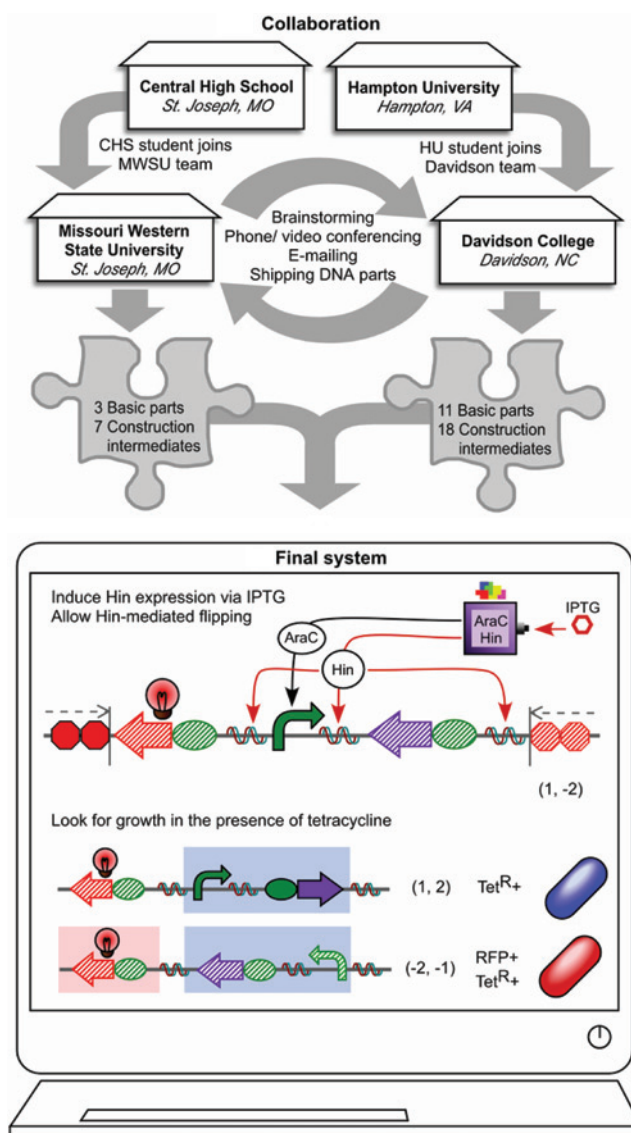


Fig. 1 Engineering living hardware via multi-institutional collaboration

During the summer of 2006, a senior from Central High School and an undergraduate summer research fellow from Hampton University were hosted by MWSU and Davidson College, respectively. Students and faculty at MWSU and Davidson College used a variety of media to develop project ideas and to execute research.

Final living hardware system consists of two plasmids: (1) an AraC/Hin generator (purple box) that encodes the AraC and Hin invertase proteins (white circles); and (2) a two-pancake stack in which the *pBAD* promoter (green bent arrow) and a tetracycline resistance coding region (purple arrow) with an upstream ribosomal binding site (green oval) are flanked by *hixC* sites (wavy lines). Pancakes in the reverse orientation are hatched. IPTG induces *pLac*-driven Hin-LVA expression; Hin-LVA recognises the *hixC* sites (red vertical arrows). AraC binds to *pBAD* and pauses transcription during Hin-mediated flipping. Forward and reverse terminators (red octagons) in cloning vector *pSB1A7* block transcriptional read-through from the backbone into the pancake stack. *E. coli* cells are co-transformed with an unsorted pancake stack (i.e. $(1, -2)$ shown here) and the AraC/Hin generator plasmids, then screened over time (increasing numbers of flips) for tetracycline resistance and RFP expression.

to the RE, or single vs. multiple pancakes. Furthermore, plasmid copy number influences the probability of randomly solving the problem in a single cell. Thus far, we have used MATLAB to simulate cell survival after completely random flipping on a single-copy plasmid; continued work will generate simulations that consider flipping bias and high plasmid copy number. Data from these simulations will help us to interpret the behaviour of Hin-mediated flipping *in vivo*.

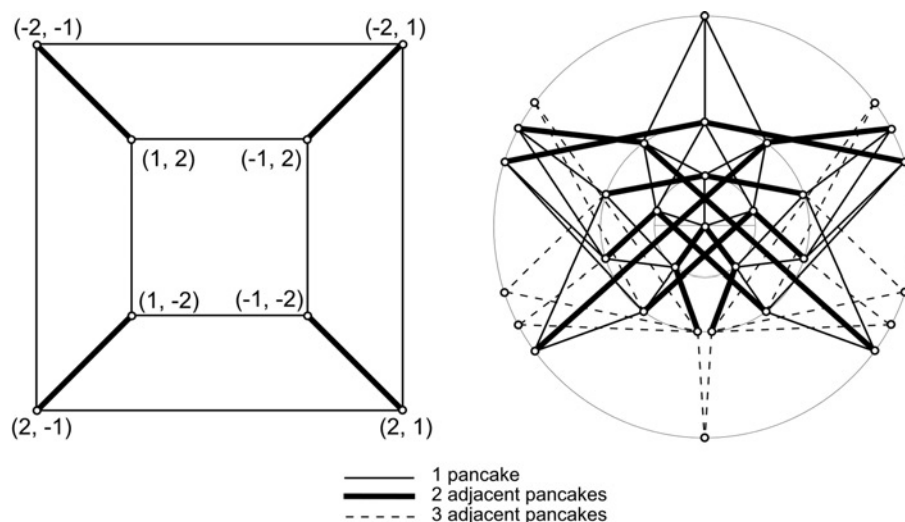


Fig. 2 *Mathematical modelling of burnt pancake flipping*

Graphs representing the relationships between signed permutations of pancake stacks. Each permutation is located at a vertex (open circle) and the edges denote flips of a single pancake or multiple adjacent pancakes. The graph for a two-pancake stack (left) has eight vertices. The graph for a three-pancake stack (right) with 48 vertices is plotted on a sphere. The 17 vertices in the Northern Hemisphere and 14 vertices on the equator are shown.

3 Discussion

3.1 *E.HOP computer is a proof of concept for computing in vivo, with implications for future data storage devices and transgenic systems*

A two-pancake stack is easy to solve without the aid of a computer, but as the stack gets larger, the puzzle becomes more computationally challenging. The parallel processing capacity of the E.HOP computer should allow us to tackle larger problems with ease. The E.HOP computer also has potential to serve as a novel means for data encoding and storage. A series of DNA pancakes arranged in either the forward or backwards orientation is analogous to binary code (1's and 0's). Sorting by reversals generates $(2^n \times n!)$ configurations of n pancakes, a combinatorial

explosion of data that could be written to plasmid 'hard drives' *in vivo*. Our BioBrick-compatible Hin/hix recombinase system may also prove useful for basic biological research. Controlled reversals *in vivo* would allow orientation-dependent function of DNA elements to be tested at a single locus. Large pancake stacks could serve as model systems to gain insights into gene rearrangements within syntenic chromosomes over evolutionary time. Furthermore, Hin-mediated genetic toggle switches could allow adjustable expression in transgenic organisms.

3.2 *Collaboration between underrepresented institutions yielded world-class research*

In multi-institutional collaborations, efficient communication is the most significant limitation imposed by a lack of direct contact between team members. Creative thinking, teamwork, and electronic communication helped narrow the physical divide between the MWSU and Davidson College campuses. Overnight express shipping, e-mail, iGEM Wiki page editing [7, 8], online instant messaging, and conferences via phone and the internet allowed us to engineer our genetic devices in sync. Despite the lack of any face-to-face meetings until the 2006 iGEM Jamboree (Fig. 3), the MWSU and Davidson College teams produce two well-meshed presentations; one of these earned a 'Best Presentation' award (MWSU, first place) [9].

This year, only three primarily undergraduate institutions (PUIs) in the USA and two minority serving institutions (MSIs) were represented among many large research-driven universities located across the globe. Our team represented about half of the underrepresented institutions (MWSU (PUI), Davidson College (PUI) and Hampton University (MSI)). It is worth noting that compared to research-intensive institutions, our schools have no graduate students, small budgets and heavy faculty teaching loads. Team member cooperation and iGEM program resources mitigated these challenges and enabled us to conduct cutting edge research that blends biology and mathematics. Our collaborative research won five awards: 'Best Presentation' (MWSU, first place), 'Best Part' (Davidson,



Fig. 3 *Team photo*

Front row (left to right): Trevor Butner, Brad Ogden, Eric Jessen; middle row (left to right): Todd Eckdahl, Laurie Heyer, Karmella Haynes, Lane Heard, Samantha Simpson, A. Malcolm Campbell, Jeff Poet; back row (left to right): Adam Brown, Marian Broderick, Sabriya Rosemond, Kelly Malloy, Lance Harden, Erin Zwack

second place), ‘Best Cooperation and Collaboration’ and ‘Best Poster’ (Davidson and MWSU, second place) and ‘Best Conquest of Adversity’ (Davidson and MWSU, third place) [9]. We have learned that multi-institutional collaboration on synthetic biology research can be a fun and rewarding experience.

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Engineering novel synthetic biological systems

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Abstract: Engineering principles and new applications for the nascent field of synthetic biology are just beginning to be explored. Here, we report the engineering of four novel synthetic biological systems: (1) a Bacterial Dynamo, for generating electricity using modified magnetotactic bacteria on a microfabricated device; (2) Cancer StickyBots, for targeting and destroying tumour cells using engineered *Escherichia coli* cells; (3) Human Encryption, an information encoding, storage, and retrieval scheme for potential security and medical diagnostic applications; and (4) X-Verter, new strategies and tools for biological circuit design and BioBrick management. While each of these systems had distinct aims, they shared a common philosophy of rationally building useful and beneficial synthetic biological systems using fundamental engineering principles. They also demonstrated the potential usefulness of BioBricks and contributed to the Registry of Standard Biological Parts and synthetic biology community-at-large.

1 Project aims

Four distinct novel biological systems were investigated for engineering principles and new applications of synthetic biology. The Bacterial Dynamo (BD) system aimed to engineer the flagella of magnetotactic bacteria so that it would adhere to and gyrate on a microfabricated metal coil substrate in a configuration that would induce a voltage and generate electricity. The Cancer StickyBots (CSB) system sought to engineer the surface and genetic payload of *E. coli* bacteria so that it could target and kill colon cancer cells in a specific and regulated fashion while the bacteria themselves are subjected to population regulation via a quorum-sensing based suicide mechanism. The Human Encryption (HE) system envisioned the use of engineered *E. coli* bacteria carrying encrypted messages and red-shifted luciferase reporters in their genetic makeup inside host mammals for potential security or medical diagnostic applications. The X-Verter (XV) system explored design strategies for complex, interdependent biological circuits and developed software and database tools for BioBrick management.

2 Results and conclusions

2.1 Bacterial Dynamo

This bioenergy conversion system involved bioengineering magnetotactic bacteria to adhere to and spin on a microfabricated metal coil, thereby inducing a voltage—in essence, converting biomechanical power of microorganisms into electricity (Fig. 1a). This system relied on Faraday's law of induction; that is, the induced electromotive force is proportional to the rate of change of the magnetic flux through a conductive coil. The strategy for surface anchoring of magnetotactic bacteria was through engineering of a flagella protein (Fig. 1b) to confer high binding affinity to positive photoresist on the surface of the gold coil.

The magnetotactic bacterium of choice was *Magnetospirillum magneticum* AMB-1 [1]. AMB-1 has flagella that confer rotary motion, and the highly conserved flagellin gene in AMB-1, *amb0684*, has been identified [2]. However, the particular structural surfaces of the flagellin protein that are exposed to the outside environment remain elusive. To facilitate engineering of flagellin in AMB-1 [3], which is anaerobic and slow-growing, a surrogate *E. coli* strain GI826 was employed to carry and transfer plasmid vectors via conjugation. In order to select peptide sequences with high binding affinity to photoresist substrate, a random peptide display library, FliTrx (Invitrogen), which contained 10^8 random 12-amino acid residues in constrained loops of modified Thioredoxin 1 (trxA) [4] was inserted into the central variable region of *fliC* [5]. The modified *fliC* gene containing the peptide display library was then cloned into the plasmid pFliTrx and transformed into a *fliC* knockout *E. coli* strain GI826. Bacteria that specifically bound to the photoresist substrate were harvested and the binding peptide sequence determined. The most promising peptide sequence was found to be 'GHRAAYRIQLR', which appeared multiple times. Furthermore, culturing of AMB-1 with desirable attributes was visually confirmed for cell colonies having dark brown magnetosomes (Fig. 1c).

The gold coil was microfabricated on scales amenable to field-effect induction by the magnetosome crystals, by

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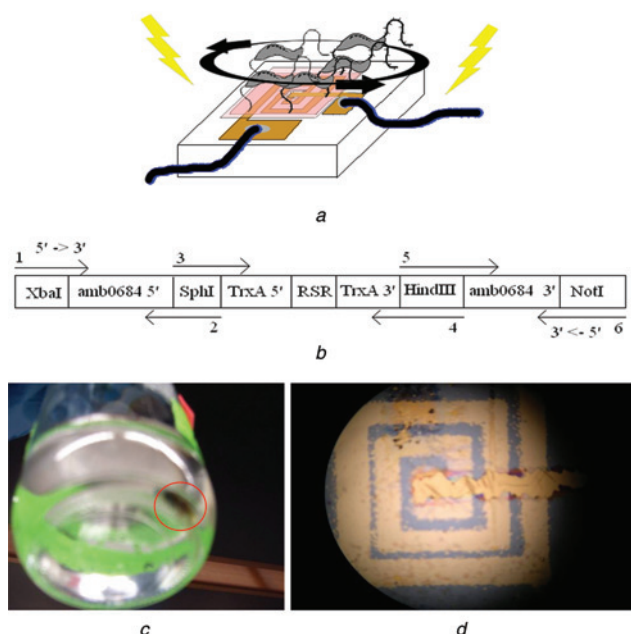


Fig. 1 Bacterial Dynamo system

- a Schematic of the Bacterial Dynamo design
b Schematic of sticky flagellin cDNA in BioBrick format
c Collected AMB-1 culture indicated by red circle
d Microfabricated gold coil (0.5 cm²)

successive coatings and patternings to a clean silicon wafer having a circular diameter of 10 cm, and with a 0.5 cm² coil having electrode contacts. To this wafer, a 300 Å layer of chromium and a 500 Å layer of gold was evaporated and developed. On top of the gold coil an insulating coating of Shipley Microposit S-1813 Positive Photoresist liquid was applied. On top of this, another similar chromium-gold layering was applied to provide the second electrode contact and to which a final layer of Positive Photoresist liquid was applied to allow binding of the engineered AMB-1 bacteria. The resulting photoresist layer was characterised to be 1.5–1.6 µm in thickness. This circular wafer with positive photoresist was then hard-baked at 121°C for 30 mins, followed by post-treatment with MF-319 developer for 1 min, to yield a positive photoresist substrate (Fig. 1d).

Success with both the bioengineering of GI826 and AMB-1 and electrical engineering of the coil apparatus demonstrates readiness for future integration of the approaches. Specifically, co-culturing should occur between GI826 and AMB-1 carrying the magnetosomes to allow transfer of the plasmids that confer the adherent peptides to photoresist substrate. Given proper conjugation and selection, this should yield an AMB-1^{adherent} strain that carries both the aforementioned properties.

2.2 Cancer StickyBots

This novel therapeutic system sought to use bacteria with three specialised biological circuits to treat colon cancers without adversely impacting neighbouring healthy cells (Fig. 2a). The three biological circuits of interest are for targeted localisation (Fig. 2b), discriminate killing (Fig. 2c), and regulated suicide (Fig. 2d). In contrast to other modalities which attempt to limit the chemotherapeutic impact to microscale tolerances and require constant administration that reduce quality of life, this system may achieve better results by being more focused to cellular nanoscales and acting in synchrony.

The three biological circuits harnessed various biological phenomena for achieving their goals. For example, in order

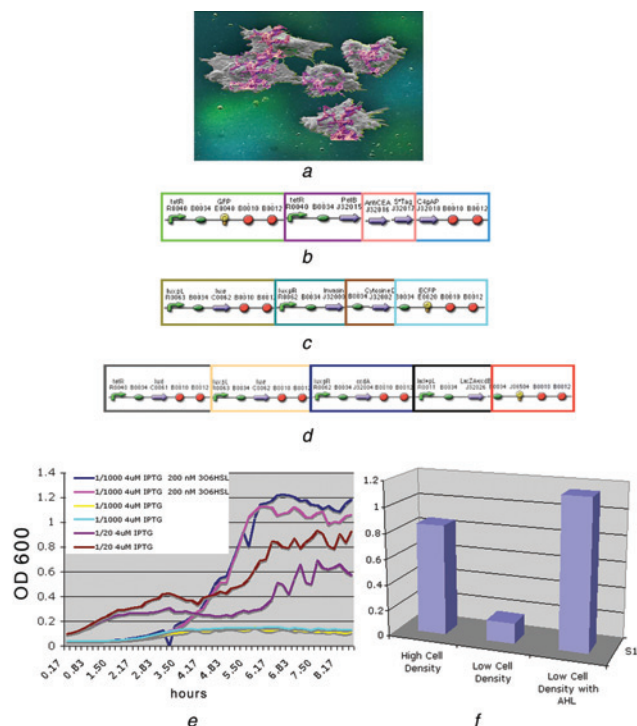


Fig. 2 Cancer StickyBots system

- a Schematic of the Cancer StickyBot design
b Schematic of targeted localisation cDNA in BioBrick format
c Schematic of discriminate killing cDNA in BioBrick format
d Schematic of regulated suicide cDNA in BioBrick format
e Optical density time course of bacterial populations performing regulated suicide in presence of IPTG and 3OC6HSL
f Cell density-dependent growth of bacterial populations highlighting recovery under influence of AHL

for the bacterial cells to perform targeted localisation to colon cancer cells, a biological circuit incorporated a C-IgAP autotransporter surface display to provide a modular scaffold [6] for the detection of carcinoembryonic antigen (CEA), which is highly expressed in colon cancer cells [7]. In order to detect this display, an Ablynx[®] cAb-CEA5 Nanobody[™]; that is, the smallest functional fragment of a naturally occurring antibody, and a S*Tag Fusion protein were also expressed on the surface. A PelB leader sequence was utilised to direct migration of proteins to the periplasmic membrane of *E. coli*. Once localised near colon cancer cells, the bacteria are designed to perform discriminate killing with the toxic small molecule 5-Fluorouracil [8]. To limit unintended toxicity, another biological circuit carried a cytosine deaminase gene that would convert the inert 5-Fluorocytosine small molecule into this toxic killer. In order to introduce this converted toxic 5-Fluorouracil into mammalian cells, an invasin gene [9] was incorporated. Both of these payloads were under quorum-sensing regulation, so that dose-dependent toxicity and invasion only occurred at a critical density of bacterial cells congregating around colon cancer cells, and not by the stray bacterial cell that failed to perform targeted localisation. A third biological circuit that performs regulated suicide aimed to eliminate those bacterial cells that failed to perform proper localisation. In addition to expressing genes responsible for synthesising and sensing a quorum sensing signal, 3-oxohexanoyl-homoserine lactone (3OC6HSL), the bacteria also contained the gene to express a toxic CcdB protein [10] under control of a Lac promoter. In the presence of IPTG, the CcdB protein was expressed, which in turn inactivated the DNA-gyrase complex and thus the passage of polymerases to repair

double-stranded breaks in DNA. Bacteria at high densities are rescued from this fate by the antidote CcdA protein that is under quorum-sensing regulation. Thus, the low bacterial cell densities failed to trigger the quorum-based rescuing pathway, leading to the suicide of these therapeutic bystanders.

The strategy of using cell–cell communication to regulate cell death has been shown to be effective in programming robust population dynamics [11]. Building on this foundation, performance of the regulated suicide circuit has been encouraging. For example, an optical density evaluation of bacterial cells under regulated suicide demonstrated that those which were administered 4 μ M IPTG as well as 200 nM 3OC6HSL were rescued and thrived while those that were given only 4 μ M IPTG exhibited diminished or insignificant growth (Fig. 2e).

Furthermore, another quorum-sensing mechanism responsive instead to an acyl-homoserine lactone derivative was investigated and confirmed density-dependent growth, with the optical density of the those cell treated with this small molecule to be similar to that of high density rather than low density cell populations (Fig. 2f). The BioBricks that permit use of these three biological circuits have been contributed to the Registry, and may form the basis of an integrated approach in the future.

2.3 Human Encryption

This proof-of-concept system involved information storage and transmission using symbiotic bacteria containing a modified bioluminescent operon, within a host mammal [12]. The advantage of this system is two-fold. First, the wavelengths of bioluminescence would serve as a readily detectable marker for a message incorporated into and

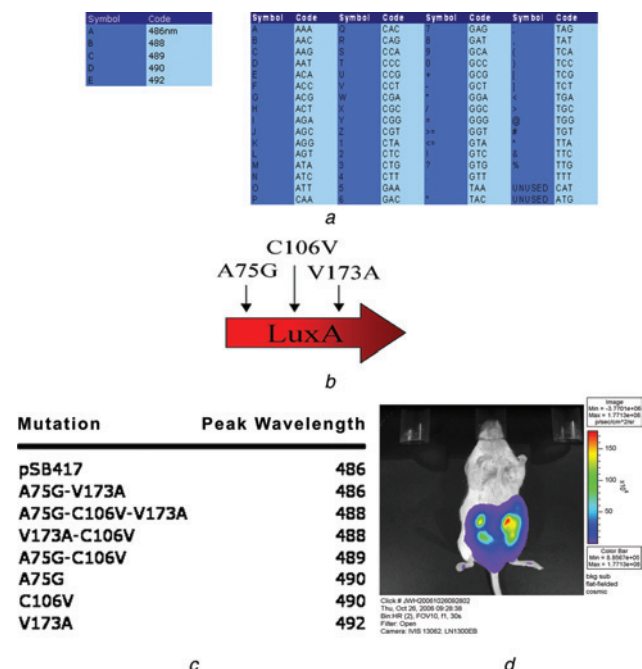
retrievable from the host in the form of DNA triplets encoding amino acids. There are 4³ possible triplets, among which 62 had been designed to represent a character set to be encrypted (Fig. 3a). Second, the wavelength of bioluminescence after mutations would be red-shifted and permit detection of bioluminescence from bacteria located in deeper tissue [12]. In order to achieve emission of bioluminescence with different wavelengths, site directed mutagenesis was performed on plasmid pSB417. A library of LuxA mutant constructs [13] were designed (Fig. 3b) via single, double and triple amino acid mutations. The constants were evaluated by measurement of peak wavelength shift.

Preliminary experiments detected a maximum red shift of 6 nm in the intensity peak for clone V173A; that is, a valine to alanine amino acid substitution at position 173 (Fig. 3c). These results are consistent with models of homologous luciferases [15]. Furthermore, *in vivo* administration and bioluminescence imaging was achieved using DH5 α *E. coli* cells with different red-shifted luciferases in mice [16]. It was observed that the bioluminescent bacteria moved from the stomach and settled in the lower digestive tract where they grew while emitting the encrypted message over a period of 17 h. (Fig. 3d).

These promising milestones motivate further elaboration of this system, with potential payoffs in understanding structure–function relationships between luciferase mutations and peak luminescence wavelengths, as well as contribution of a library of luminescent BioBricks to the Registry. Further foreseeable applications include a ‘bacterial’ biosensor that emits certain wavelengths based on sensed conditions in the gut as well as storing and retrieving messages in biological systems.

2.4 X-Verter

This innovative system harnessed a quorum-sensing strategy [17], similar to what was described earlier, coupled to



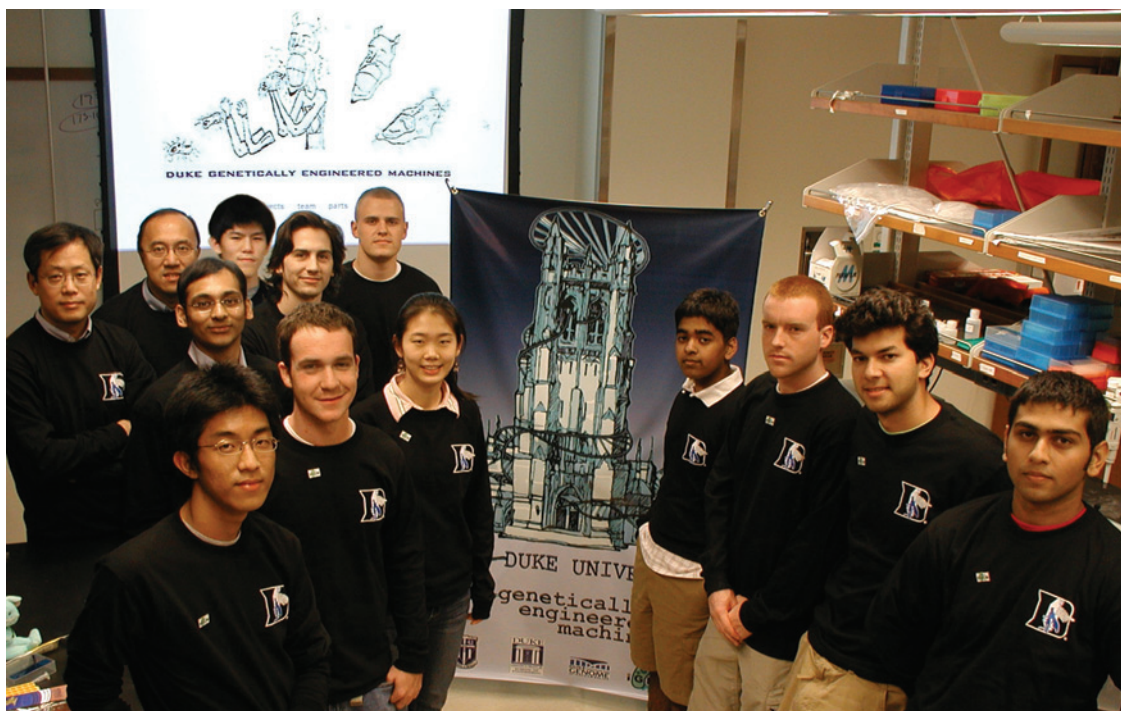


Fig. 5 *Duke University Genetically Engineered Machines 2006 Team*

Left side, front row, left to right: John Lee, Eric Josephs, Hattie Chung
 Left side, middle row, left to right: Faisal Reza, Austen Heinz, Bryan Van Dyke
 Left side, back row, left to right: Jingdong Tian, Fan Yuan, Nicholas Tang
 Right side, left to right: Sagar Indurkha, Pat O'Brien, Keddy Chandran, Nirav Lakhani
 Not pictured: Matt Feltz, Thomas LaBean, Steven Lin, Lingchong You

gene repression [18] in order to create biological circuits that act as multistage interdependent synchronised synthetic biological oscillators (Fig. 4a). Unlike related notable achievements, such as the repressilator [19], this system aimed to synchronise the oscillations through quorum-sensing at a population rather than single-cell level, thereby reinforcing the presence of oscillations over the intrinsic noise and stochasticity [20], extending their numbers beyond a few, and do so in a modular and easily extensible fashion. It did so by an X-Verter receiver reporter biological circuit (Fig. 4b) that responded through fluorescence to an X-Verter sender device (Fig. 4c) that processed the synchronisation inputs and outputs.

Here too, the biological circuits involved took advantage of natural cellular processes to achieve the goals. The specific properties of the quorum-sensing method and associated small molecules determine signal transfer and

molecular crosstalk. Since these are poorly described in the literature, degradation properties of four small molecules HSL derivatives [21], $C_{18}H_{31}O_4N$, $C_{16}H_{27}O_4N$, $C_{10}H_{15}O_4N$, and $C_8H_{13}O_3N$ for the Cin, Las, Lux, and Rhl systems, respectively, were performed using computational chemistry techniques. The Gaussian 03 (Revision C.02, Gaussian Inc.) software calculated molecular orbitals and infrared spectra for these derivatives with a restricted Hartree-Fock ground-state approximation using the 6-31G base set. This showed that the HOMO (highest occupied molecular orbital)/LUMO (lowest unoccupied molecular orbital) gap were similar among these four AHL derivatives, thus predicting similar degradation or hydrolysis rate potentials.

A theoretically plausible method for synchronisation through inducible gene regulation and population sensitive quorum-sensing was mathematically modelled using empirical and predicted values for kinetic parameters to yield stable oscillations as seen in the X-Verter reporter time-course (Fig. 4d). This is similar to the behaviour expected from a three-stage receiver reporter circuit from a sender device circuit (Fig. 4e).

This synchronisation suggests that it is possible to overcome barriers such as limited cell lifetime and naturally induced noise and regulate the discrete operation of the circuit at the population level. In doing so, the X-Verter provides an additional motif for controlling genetic logic and function among plasmids and cells. Artificial oscillating populations demonstrate the potential for larger and more complex synchronised genetic circuits, such as the coupled oscillatory behaviour. These may eventually shed fundamental insight on natural biological systems and be applied towards effective gene delivery, drug and metabolic platforms, as well as provide temporally sensitive sender and receiver BioBricks to the Registry in the short-term.

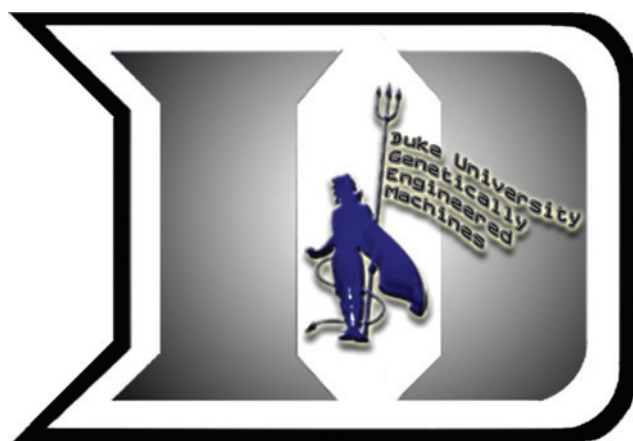


Fig. 6 *Team logo*

3 Acknowledgments

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Design of a biological half adder

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Abstract: The building of complex systems from basic logic gates is one of the hallmarks of circuit design in electrical engineering. The question arises whether a similar strategy can be adopted for the design of artificial biological systems. In this paper, we present the design of two logic gates, a biological AND and a biological XOR. They can be combined to produce a half-adder, one of the fundamental elements of complex systems engineering, and represent a promising basis for the design of more complex genetic circuits. Design space exploration allowed us to screen gate variants, while sensitivity analysis of refined models contributed to the specific implementation of the gates at the DNA level. The XOR gate is based on two specific proteases, which reciprocally inactivate co-synthesised transcription factors. The AND gate is designed such that, in the presence of two signals, a tRNA suppresses the premature termination of T7 RNA polymerase translation. Computer models confirmed that both designs allow gate behaviour that is reasonably close to idealised gates.

1 Introduction

Naturally occurring biological systems integrate complex sets of information and process these to perform complicated actions. In contrast, current artificial biosystems such as oscillators, switches, or logic gates [1] are rather simple and have limited functionality. However, simple functions such as logic gates can be used as modular building blocks. This approach has allowed engineers to produce digital electronic systems with the enormous complexity of modern microprocessors. Consequently, we aimed at designing and implementing a more complex biological functionality from simple logic gates. One standard and versatile function that is well established in electric circuit engineering is the half-adder that is itself composed of two logic gates, an AND and an XOR gate. Such a half-adder leads to one output if one of two signals is present and to another output if both signals are present simultaneously. In this communication, we report the design of an artificial biological counterpart.

2 System overview

An electronic half-adder accepts two single digit binary inputs and produces a two bit-sum as the output. The more significant bit of the output is called the carry-over, in analogy to the addition procedure on paper, where this number is carried over to the next position. In the binary half-adder, three cases can be distinguished: first, both inputs can be 0. In this case, the sum is 00 and the carry-over is 0. In the second case, one of the inputs is 1 and the other is 0, so the sum is 01 and the carry-over is still 0. Finally, if both inputs are 1, the sum is 10, and the carry-over is 1.

The less significant bit of the sum is the result of an XOR logical operation on the inputs, resulting in 1 only when exactly one of the operands is 1 and 0 otherwise. The more significant bit of the sum is the outcome of an AND operation on the inputs, which is 1 only when both inputs are 1 and 0 otherwise. The constituent gates of the half-adder are operating in parallel, thus the implementations can be designed independently.

In a biological half adder, any two stimuli representing the inputs, for example, presence of light and/or chemicals, are converted into two biological signals (e.g. enzyme activity or regulatory gene expression), which then are processed by the system. The outcome of this is either the absence of a reporter protein, RFP synthesis (one signal present, value of 1 in the output of the XOR gate), or GFP synthesis (both signals present, value of 1 in the AND output) (Fig. 1a). Depending on the chosen implementation and integration of the gates, additional measures might be necessary to connect a single input to two logic gates. The signal duplexer serves this purpose in our design.

3 Exploration of design space

The advantages of mathematical modelling include the identification of parts that contribute most to properties of interest and faster and cheaper generation and testing of hypotheses [2]. Successful system design requires several iterations of modelling, testing, model selection, and refinement. Early iterations focus on design space exploration (Fig. 1b), where the number of system design alternatives is reduced based on several cycles of simulation and optimisation—and of course the consideration of biological feasibility. At later stages, models can be further improved by accounting for experimental data. We focus here on the design space exploration.

The modelling was carried out separately for the XOR and the AND part. Various concepts to design biological XOR or AND gates are theoretically possible, a selection of which is collected in Fig. 2. Simulations of these models were performed to identify a suitable steady-state behaviour. Furthermore, the models were analysed for parameter sensitivity to identify relatively robust designs.

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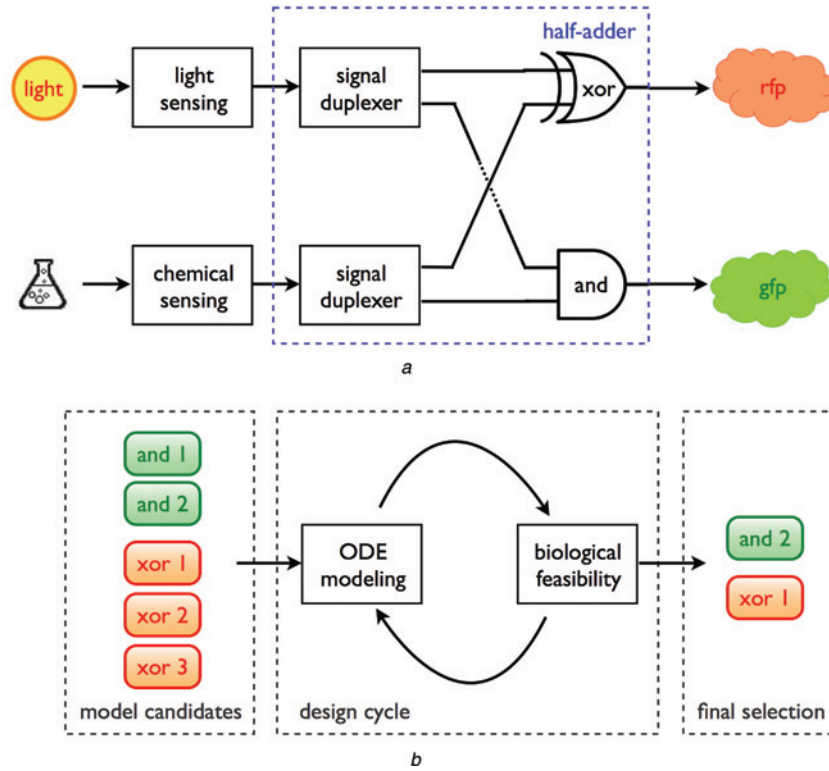


Fig. 1 Schematic overview of a biological half-adder system

a Two biological stimuli, light and a chemical signal, are sensed by respective devices. The duplexers direct the input signals to XOR and AND gates. In the case when one of the two inputs is present, the XOR gate is activated and produces RFP. When both inputs are present, the AND gate is activated and produces GFP. Lack of both inputs results into the absence of both reporter proteins

b Engineering design approach to model selection: several AND and XOR model candidates are generated, based on abstract notions of possible implementation. Through iterations of ODE modelling and biological feasibility considerations, relatively better models are selected to be experimentally tested

Finally, the models were analysed for feasibility of biological implementation. The corresponding MATLAB scripts are available from the authors upon request.

3.1 ODE systems

Ordinary differential equations (ODEs) were used for modelling the gate variants. Altogether, the models contain four different types of ODEs describing enzymatic transformation, constitutive transcription, regulated transcription, and translation (see online supplementary material). For example, the equations below illustrate the system of ODEs for the final AND gate

$$\begin{aligned} \frac{d[T7Pol]}{dt} &= k(tl)_{T7Pol}[mT7Pol] \\ &\quad \times [tRNA] - d_{T7Pol}[T7Pol] \\ \frac{d[T7Pol^*]}{dt} &= k(tl)_{T7Pol^*}[mT7Pol] - d_{T7Pol^*}[T7Pol^*] \\ \frac{d[tRNA]}{dt} &= k_{tRNA}[Input1] - d_{tRNA}[tRNA] \\ \frac{d[mT7Pol]}{dt} &= k_{mT7Pol}[Input2] - d_{mT7Pol}[mT7Pol] \\ \frac{d[mOut]}{dt} &= p_{Out} + \frac{k(tr)_{Out} \left(\frac{[T7Pol]}{K_{Out}} \right)^{n_{Out}}}{1 + \left(\frac{[T7Pol]}{K_{Out}} \right)^{n_{Out}}} \\ \frac{d[Out]}{dt} &= k(tl)[mOut] - d_{Out}[Out] \end{aligned}$$

The bracketed species above refer to concentrations, and the symbols are detailed in the legend of Fig. 3.

3.2 System selection

Fig. 2 shows surface plots for the different models corresponding to different design alternatives. The output rates for the gates as a function of two inputs at steady state after 12 h simulation are shown. The left and the middle column represent models that were prepared early in the model refinement process. These guided decision making and have little more than qualitative value. The right column shows final versions. These are more realistic since experimental data, available from other systems, has been used to estimate parameters such as reaction constants [3, 4].

Fig. 2a–c shows three versions of the XOR gate. An ideal XOR gate should have high output if one input is high and low output if both inputs are either low or high. Output behaviour of XOR 1 (Fig. 2a) is clearly preferable to the behaviour of XOR 2 (Fig. 2b). Combined with a simpler biological implementation, this led us to select XOR 1 for elaboration. XOR 3 (Fig. 2c) is based on XOR 1 and has been adapted to the selected biological realization (see below) and refined with realistic parameter values.

Fig. 2d–f show AND gate versions. An ideal AND gate has high output only if both inputs are high. AND 1 (Fig. 2d) shows AND-like behaviour, but the thresholds for low and high output are very close and might be impossible to distinguish in experiments. On the other

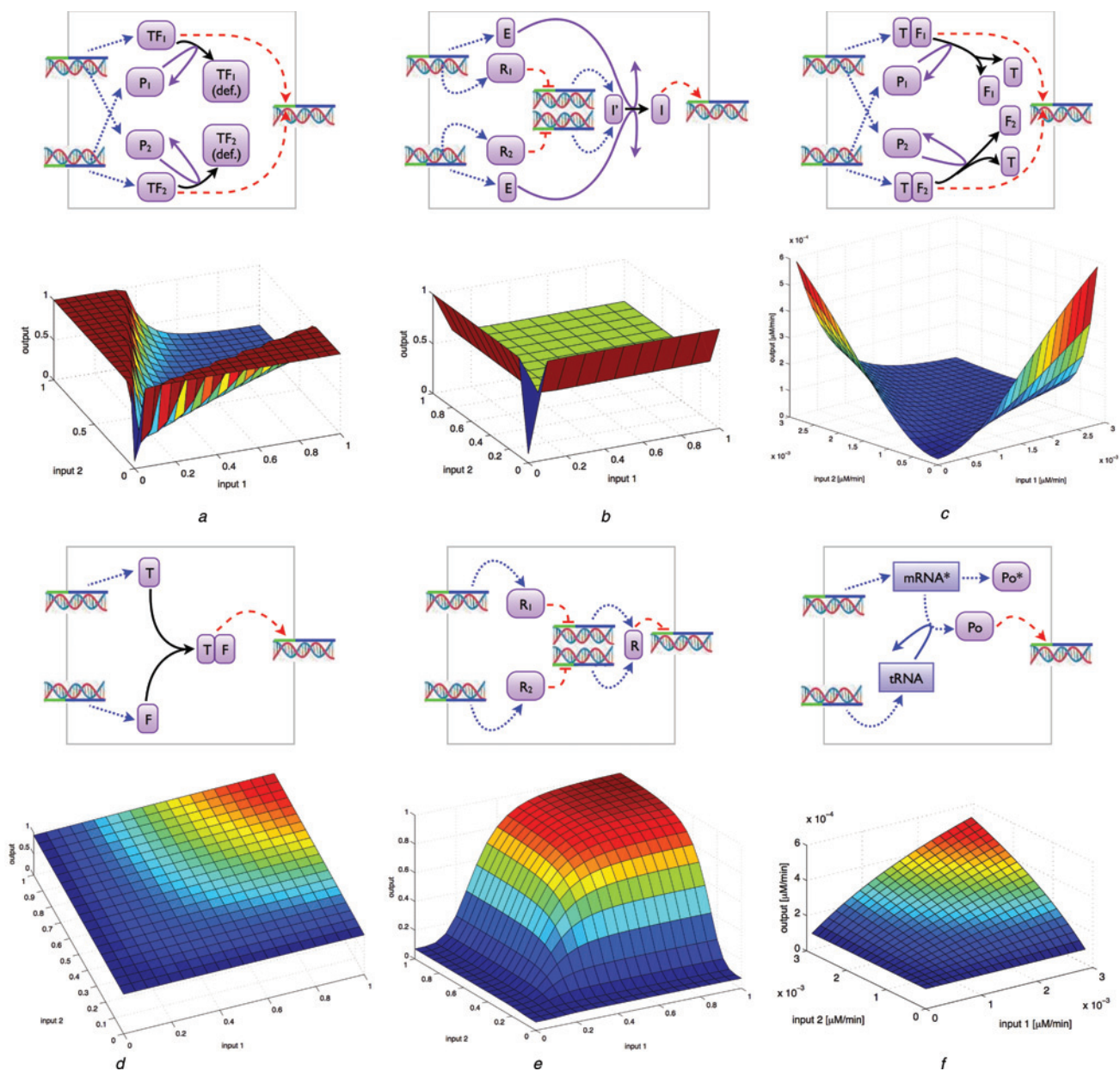


Fig. 2 Biological structure and modelling results for various design alternatives

The modelling results in the second and forth row illustrate the steady-state output of the system (z-axis) as a function of two varying inputs (x- and y-axis) for the biological system illustrated above. The green parts of the DNA elements represent regulatory, the blue part coding sequences. Dotted blue arrows indicate protein expression; solid black arrows represent enzymatic reactions, enzyme participation shown in purple. Dashed red lines highlight regulatory interaction (arrow for induction, bar-headed for repression). Early stage models (a, b and d, e) have mainly been used to support decision making and thus have little more than qualitative grade. For this reason, normalised values are shown on the axes

a XOR 1: Input 1 triggers the synthesis of transcription factor (TF) 1 and protease (P) 2 from the upper DNA element. TF1 triggers synthesis of the reporter (XFP). Input 2 triggers synthesis of TF2 and P1 from the lower DNA element and also leads to synthesis of XFP. When both inputs are present simultaneously, P1 digests TF1 and P2 digests TF2, resulting in mutual inactivation of the transcription factors

b XOR 2: Either input triggers synthesis of a repressing TF R1 or R2 and of an enzyme E. E is needed to activate an inducing TF I' to I, and I triggers the synthesis of XFP. Only the presence of both repressors, R1 and R2, stops the synthesis of I' and prevents synthesis of XFP

c XOR 3: Final XOR model derived from XOR 1 using two very similar transcription factors, TF1 and TF2, with corresponding specific proteases P1 and P2, which inactivate the transcription factors by mutual cleavage

d AND 1: Two parts T and F of a transcription factor triggering synthesis of XFP are produced from two DNA elements. Both input signals are required for a functional TF

e AND 2: (double repression): XFP synthesis is repressed in the presence of repressor R. R can be synthesized from two DNA elements, and repression of R synthesis requires another repressor for each element, R1 and R2. Consequently, only the presence of two inputs turns on XFP synthesis

f AND 3: Input 1 triggers the synthesis of a truncated polymerase Po^* from $mRNA^*$. Input 2 triggers the synthesis of a suppressor tRNA that allows to suppress the early termination of translation. Simultaneously, inputs 1 and 2 allow the production of a full-length polymerase to trigger synthesis of XFP from its cognate promoter

hand, AND 2 (Fig. 2e) has almost ideal characteristics but is complex to implement biologically. AND 3 has high output for high inputs, but the transition from low to high output is not as steep as for AND 2. Finally, the possibility of simple and elegant implementation lead us to select AND 3.

4 Biological implementation

4.1 XOR gate

As already mentioned above, an XOR gate produces an output only if exactly one of the two possible input signals is present. This requires a system where gene

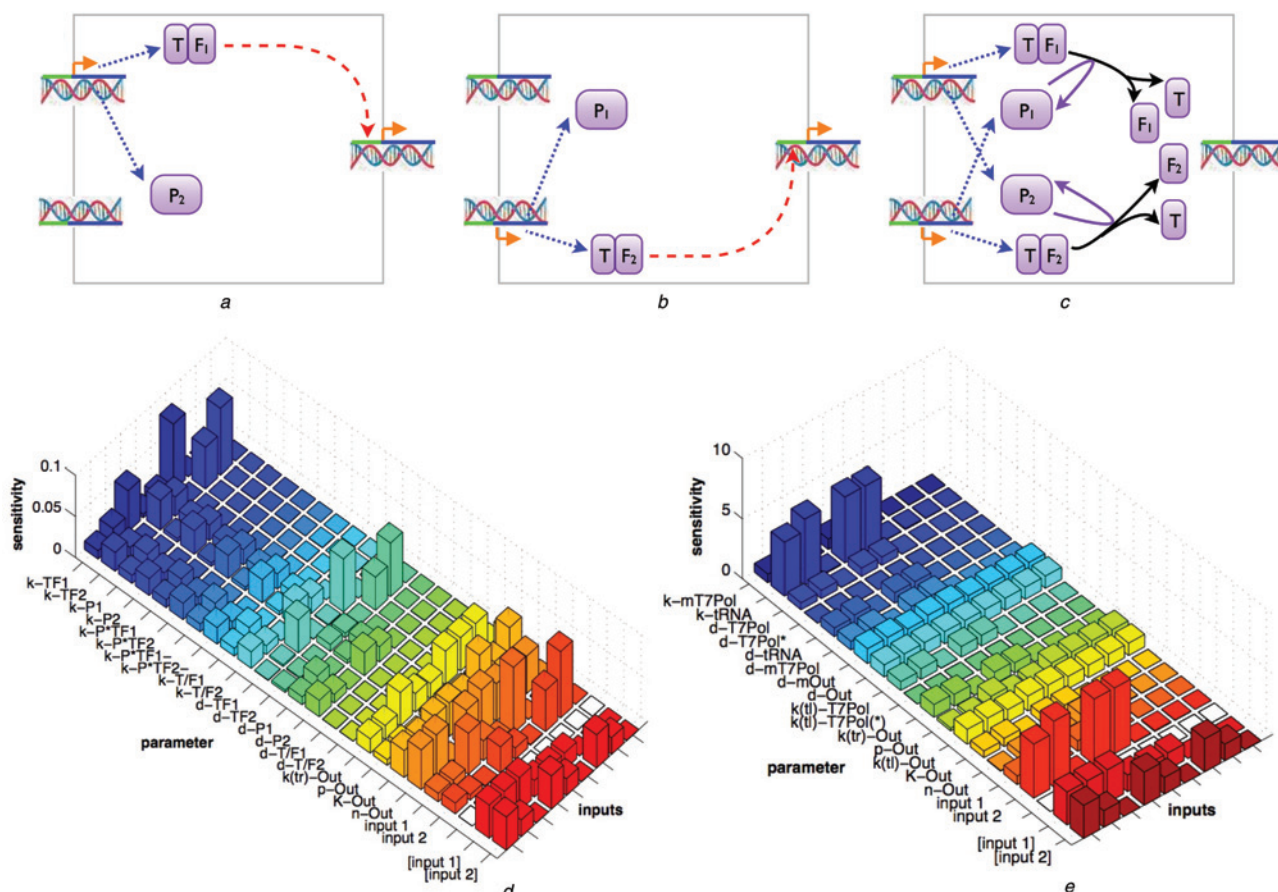


Fig. 3 XOR gate under different signal conditions (a–c); sensitivity plots of the final XOR and AND gates (d–e)

Notation as in Fig. 2; orange arrows represent triggered expression

a only Signal 1 is present—due to the incompatibility of the expressed transcription factor and the protease, reporter gene expression is induced

b only Signal 2 is present—same outcome as in a

c Signal 1 and Signal 2 are present—due to mutual cleavage of the transcription factors by the corresponding proteases, reporter gene expression is not induced

d–e The z-axis shows the normalised sensitivities. Higher bars represent stronger output sensitivity to changes of corresponding parameters. The other axes correspond to the parameters (longer axis) and nine different input combinations (all combinations of low, medium and high activity for both inputs). The two right most series [(input 1) and (input 2)] show which specific combination of inputs has been used. For example, the first row describes high input 1 and high input 2, the next high input 1 and middle input 2, etc. Note that some directly output-related parameters of the XOR gate are not displayed to enhance clarity for other parameters more relevant for the present work

The following abbreviations have been used:

(i) prefixes identify the parameter type: d = degradation constant; k = reaction rate constant; k(tl) = kinetic constant of translation; k(tr) = kinetic constant of transcription; K = Hill constant; n = Hill coefficient; p = constant background level (leakiness) of regulated transcription, typical values are 5–10 % percent of k(tr)

(ii) suffixes identify following terms: mOut = mRNA encoding the output signal (reporter); mT7Pol = mRNA encoding the T7 polymerase; Out = output signal (reporter protein); P1 and P2 = Protease; T7Pol = full length T7 polymerase; T7Pol* = truncated T7 polymerase; TF1 and TF2 = transcription factor; T/F1 and T/F2 = cleaved transcription factor; P*TF1 and P*TF2 = enzyme substrate complex in the forward direction; P*TF1- and P*TF2- = enzyme substrate complex in the reverse direction; tRNA = suppressor tRNA

expression triggered by signal 1 inhibits the gene expression triggered by signal 2 and vice-versa. We designed a system consisting of two biological units: (i) a bi-modular transcription factor with the DNA binding domain separated from the transcriptional activation domain by a protease cleavage site; and (ii) the corresponding protease with a high specificity for the protease cleavage site. Presence of the protease should lead to efficient cleavage and therefore deactivation of the transcription factor. Therefore, a functional XOR gate requires two transcription factors acting on the same promoter, but separated by different protease cleavage sites corresponding to two different and specific proteases. Signal 1 alone leads to expression of the reporter gene (Fig. 3a): First, the bi-modular transcription factor 1 with cleavage site 1 is synthesised together with protease 2. However, protease 2 cannot cleave transcription factor 1, so the downstream reporter is synthesised. The same principle applies if only signal 2 is present (Fig. 3b). This triggers synthesis of transcription factor 2, containing the protease cleavage

site 2 and protease 1. In analogy to the situation described above, transcription factor 2 is not cleaved and induces reporter gene expression. If both signals are present (Fig. 3c), the synthesis of both variants of the transcription factor and of both proteases is triggered, which leads to cleavage of both transcription factors and the reporter gene is not expressed.

Implementation of this system requires two highly specific proteases which can be synthesised in their active form in bacteria *in vivo* and preferably have no additional cleavage sites in other proteins of the host, such as the proteases from the tobacco etch virus (TEV) [5] and the tobacco vein mottling virus (TVMV) [3].

The bi-modular transcription factor consists of the DNA binding domain of the bacteriophage lambda repressor protein (λ cl) fused to the N-terminal domain of the RNA polymerase α subunit via a linker containing the corresponding TEV or TVMV protease cleavage sites ([6] and A. Hochschild, pers. comm.). Binding of this transcription

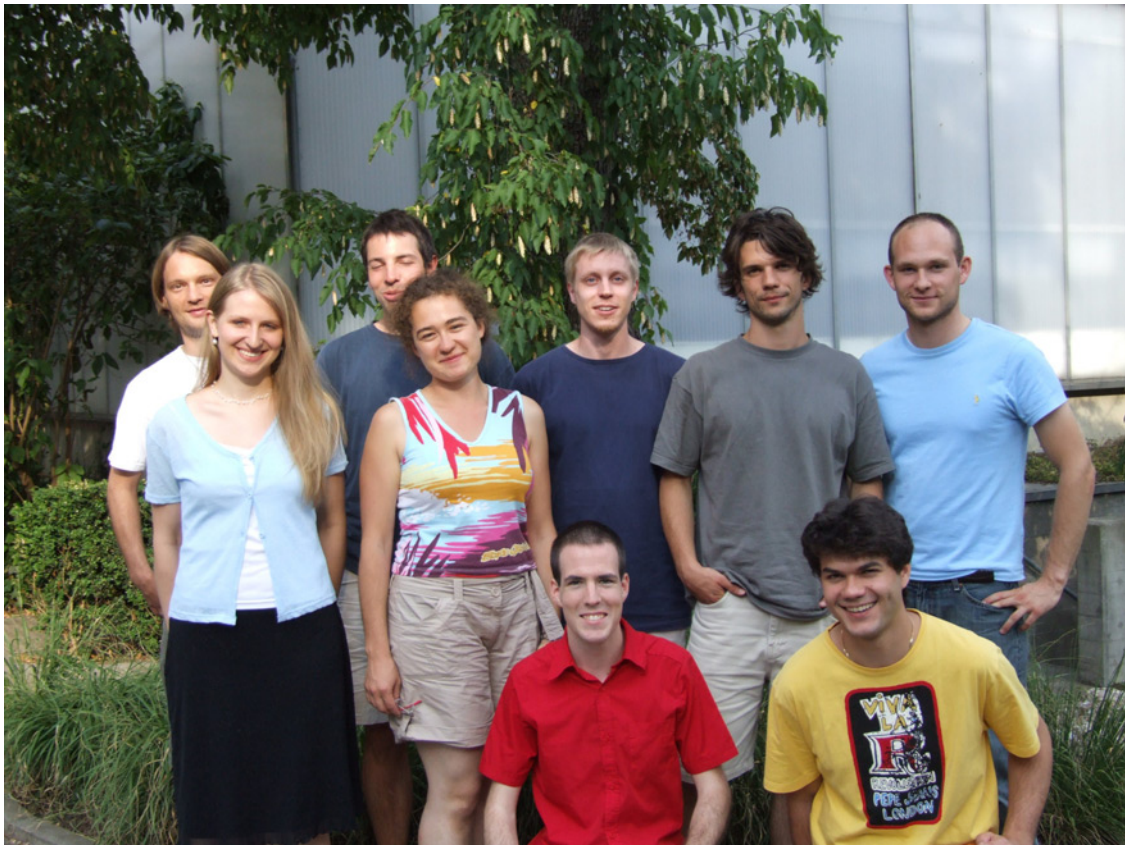


Fig. 4 The ETH Zurich 2006 iGEM team

factor to its cognate λ operator promotes transcription from a modified P_{lacZ} promoter and finally the synthesis of RFP as a reporter.

4.2 AND gate

An AND gate produces only an output if both input signals are present. This requirement is met by coupling the reporter gene expression to the presence of two essential biological units, each induced exclusively by the presence of one signal. In our design, signal 1 leads to synthesis of the T7 RNA polymerase, which recognises specifically its cognate T7 promoter [7]. An artificial early stop codon in the coding sequence of the polymerase leads to synthesis of a truncated, non-functional protein. Signal 2 triggers the synthesis of a suppressor tRNA that recognises the stop codon within the T7 RNA polymerase and prevents the premature termination of translation by incorporating a glutamine. Consequently, only the presence of both signals leads to a functional T7 RNA polymerase and expression of the downstream reporter gene (GFP) from the T7 promoter (Fig. 2f).

5 Sensitivity analysis of final designs

In silico sensitivity analysis can provide preliminary information about the expected robustness of the system in advance and help in guiding system and experimental design. We determined sensitivity as the change in output for a small change in a specific parameter or input value by computing partial derivatives of the ODEs with respect to all parameters analytically, i.e. by computing the Jacobian Matrices of the system. For details, see the online supplementary material.

The sensitivity plots confirm the model structure for both gates (Fig. 3d, e). For example, the expected insensitivity of the XOR gate output towards changes in the degradation rate of the cleaved transcription factor is confirmed

(parameters d-T/F1 and d-T/F2, Fig. 3d), and so is the insensitivity of the AND gate's output towards the degradation rate of the truncated T7 Polymerase (d-T7Pol*, Fig. 3e).

Next, the sensitivity analysis indicates that the degradation rates of the proteases in the XOR gate (d-P1 and d-P2, Fig. 3d), have a relatively small influence on the system response, which is not obvious as the proteases act catalytically and their half-life might thus have a strong influence on the performance of the system. Guided by the analysis, we refrained from shortening the protease half-life by tagging [8, 9]. Interestingly the analysis actually showed that the degradation rates of the transcription factors themselves, d-TF1 and d-TF2, are critical for the system response.

Finally, the analysis suggests that the AND gate has a strong asymmetric behaviour regarding the two input signals (Fig. 3e). It shows a much higher sensitivity toward the input triggering suppressor tRNA synthesis (input 2, k-tRNA) than toward the input triggering T7 polymerase synthesis. This asymmetry is not reflected in the simulations of the steady state model (Fig. 2f). The reason for this will be addressed in future work.

6 Implementation

Based on the results discussed above, we designed two DNA elements and deposited them in the MIT registry of standard biological parts (<http://parts.mit.edu>), one for the AND gate (registry part BBa_J34100) and one for the XOR gate (BBa_J34200) and had them *de novo* synthesised. For so far unclear reasons, reassembly of the XOR gate from subfragments into high copy number plasmids has remained elusive. Next steps will include the integration of the AND and XOR gates with sensing devices, such as the light sensing device [10] (BBa_I15008, BBa_I15009, BBa_I15010, BBa_R0082) and an IPTG-sensing device based on the LacI/ P_{lacZ} system [11].

7 Conclusion

The combination of artificial self-sufficient building blocks allows the realisation of more complex functionalities in biological systems, such as a half-adder from XOR and AND gates. Design space exploration coupled to biological feasibility assessment allows the rational selection of superior designs and sensitivity analysis of a refined model can help identify the most crucial influences in the system to direct experimental efforts.

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Biological implementation of algorithms and unconventional computing

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Abstract: The Mexican iGEM team (http://parts2.mit.edu/wiki/index.php/IPN_UNAM_2006) is a recently established group whose main interest is the implementation of algorithms in biological systems. Our goal is to take advantage of the intrinsic features of these systems in order to explore new approaches to certain computations (unconventional computing). We focus on three different frameworks: cellular automata, reaction-diffusion based computations and approaches from game theory. In the near future we plan to develop real-world applications that not only contribute to the understanding of specific problems in biology, computer science and related disciplines, but that also have a positive social impact. We are optimistic about the great benefits that genetically engineered machines might offer, particularly in a country like Mexico. On the other hand we are also conscious of the risks they involve and would like to stimulate a serious discussion about ethical and legal implications as well as the impact they might have on the community.

1 Aims of the project

The project focuses on the implementation of algorithms in biological systems. For that we have chosen three specific aspects:

- (1) Computation based on cellular automata
- (2) Reaction-diffusion implementations
- (3) Game theory problems and applications

Our group has previous experience in cellular automata, in particular through the work of G. Martínez who has extensively studied the diffusion life rule 110 [1] (see Fig. 1). Much progress has been made in exploring the possibilities of these systems in unconventional computations. More specifically, how gliders, blinkers and other structures arising in cellular automata can be used in order to implement logical gates and, eventually, more complex algorithms. One of our first goals is to be able to realise these automata in biological systems. From a more abstract point of view, we are also interested in the connection of these systems with formal languages and graph theory.

In recent years the implementation of unconventional computational techniques, specifically those based on reaction-diffusion systems have attracted interest for both theoretical and applied reasons (see [2] for example). Several computations have been successfully carried out

in real chemical systems and it seems natural to try to extrapolate them to a biological setting.

From a theoretical point of view, it is of great importance to understand the role of the architecture of genetic networks that lead to pattern formation. In particular, simple network architectures might account for the emergence of complex patterns. Currently we are working on the implementation of an activator-inhibitor model in the simple setting of a network consisting of two genes (Fig. 2) that has a counterpart in a real system [3]. We investigated whether such systems could produce so-called Turing patterns.

Finally, we also considered the possibility of designing genetic circuits coding different strategies in several classical games, such as the dove-hawk or the prisoner's dilemma. Once done, it would be possible in principle to create several types of populations of bacteria that each carried out a different strategy. This would allow us to test and further explore collective behaviour from a game-theoretical point of view. In particular it would be interesting to see if some concepts such as evolutionary stable equilibria or Nash equilibria can be obtained. This approach could lead to potentially useful applications, since modifying the strategy of the bacteria can result in a viable therapy for some diseases.

2 Description of the work

As pointed out before, iGEM Mexico is a recent group and we are still at the initial stages of our project. Right now we have met all the requirements in terms of experimental equipment as well as expertise and are beginning to develop and build our prototypes. We are hoping to present them at the next iGEM competition. We are also actively seeking financial support from our institutions as well as from other sources both public and private. Fig. 3 shows some members of the iGEM Mexico team who

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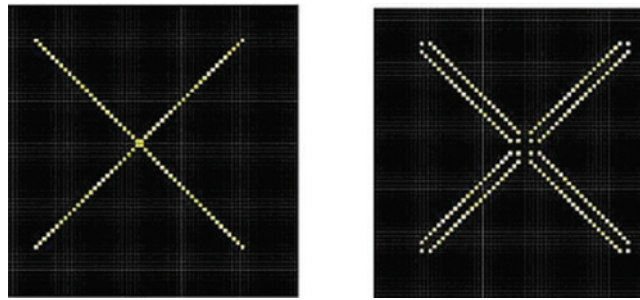


Fig. 1 An example of a configuration obtained with the diffusion rule

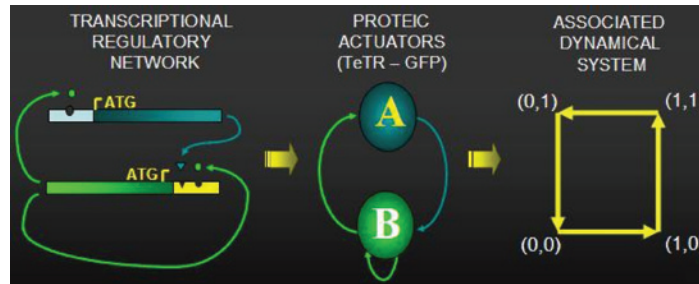


Fig. 2 Schematic representation of a simple transcriptional regulatory network leading to an activator-inhibitor system and its dynamical systems counterpart



Fig. 3 Several members of the iGEM Mexico team in the 2006 Jamboree at MIT

From left to right are Paulina A. León Hernández, Jaime López Rabadán, Tania Bermúdez Cisneros, Randy Rettberg, Carlos Silva Sánchez, Fabiola Ramírez Corona and Rosaura Palma Orozco

participated in the iGEM2006 Jamboree 2006 at MIT and received from Dr Rettberg the 'Best Work in Progress' award.

3 About the group

The iGEM Mexico team started around March 2006 when Randy Rettberg and Genaro Martínez began to explore the possibility of forming a Mexican group. In July the iGEM ambassador for Latin America, Meagan Lizarazo, visited Mexico City. By then several students and researchers from the IPN (the National Polytechnic Institute) and UNAM (the National Autonomous University of Mexico), including biologists, computer scientists, physicists and mathematicians had put forward a specific program based on unconventional computing.

4 Conclusions

We have been able to successfully integrate a group of students and researchers in order to start iGEM Mexico. We have also proposed concrete projects based on the implementation of algorithms in biological systems which focus on:

- Cellular automata, in particular the rules of diffusion needed to carry out unconventional computations
- Connections with formal languages and graph theory
- Pattern formation generated by simple genetic regulatory networks and its possible connection with Turing patterns.

Eventually, we would also like to investigate:

- Genetic algorithms in real biological systems
- Information processing with genetically engineered machines
- Distributed data bases
- Virtual reality applications.

5 Acknowledgments

We would like to gratefully acknowledge the support and encouragement from iGEM at MIT, particularly from Meagan Lizarazo. The UNAM participants would also like to thank the financial support from UNAM, through the project PAPIIT IN113406/2 Lenguajes Formales en Sistemas Biológicos.

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Synthetic sports: a bacterial relay race

J.P. Badalamenti, L.E. Weiss, C.J. Buckno, T.L. Richard, P.S. Weiss and P.C. Cirino

Abstract: Pennsylvania State University is well known for the breadth and depth of its athletic programs. But, until last year, the Penn State Athletic Department had focused on tuition-paying and scholarship-funded eukaryotes, while bacteria had languished in neglect. To reconcile this apparent inequity, we designed a bacterial relay race. We eventually hope to have a multi-leg relay race with laps, but for the initial iGEM competition the project was simplified to a ‘hand-off’ where a group of motile bacteria would encounter an immotile group, transferring a signal to turn on the latter’s motility.

1 Introduction

The bacterial relay race takes advantage of an ability to control cellular motility using inducible promoters such as those involved in nutrient catabolism or quorum sensing. ‘Receiver’ bacteria move in response to small-molecule signals either added to the system or originating from motile, ‘sender’ strains. The most significant challenges relating to this project stem from difficulties of tightly controlling the target motility gene *motB*. Low levels of *motB* expression result in system failure (constitutive motility), and resolving this issue is essential to developing reliable modular systems that are the hallmark of synthetic biology. The principal applications of this project relate to using a low concentration of a signal to activate an otherwise tightly repressed gene. Sensitive signal reception is essential for cell-to-cell communication where dilution and membrane barriers reduce inducer concentrations for target gene regulatory elements. Incorporation of effective activation devices could increase the efficiency of many systems including drug production in microbes [1]. Other potential applications of this project relate to motility and cell-to-cell communication. The ability to switch motility on and off would allow the use of bacteria as message carriers in many microfluidics applications, ranging from chemical sensing to drug delivery.

After initial deliberations, the Penn State iGEM team settled on pursuing a functional bacterial relay race. Our relay race had several primary requirements. First, a signal must be transferred from the motile bacterial strain (‘sender’ cells) to an immotile strain (‘receiver’ cells). Motility in the receiver must then be switched on with a short response time and specifically in response to the

signal of interest. For the relay race to stay on track, we also wanted to direct the movement of the newly motile cells once the signal has been received.

In choosing the signalling method to communicate between the two sets of bacteria, we explored quorum sensing as well as bacterial conjugation. Bacterial conjugation was considered as a mechanism for the transfer of a signal from one strain, to enable motility in another. However this was ultimately dismissed because the conditions required for conjugation conflicted with our probable setup for motility; simultaneously attempting to prepare the cells for both processes would shift conditions too far from optimal [2]. Instead, there was no such conflict with diffusion of a chemical signal. Quorum sensing has previously been used to regulate circuits [3] and could offer a fast method of signalling between the two cell groups.

When choosing a method to control motility, our primary concern was how quickly motility could be restored once the signal was received, and also how we could keep the receiver cells immotile before they were activated. The first option we explored was the well-studied *Che* transduction system. Parkinson showed that by lowering expression of *cheY* (or *cheA*), bacteria exhibit comparatively larger running times, while bacteria with *cheZ* deletion were deficient in linear movement and exhibited only tumbling behaviour [4, 5]. Through varying the expression of the *cheY* gene, mutants favouring tumbling or running would be observed [6]. The second approach, which was selected for its ability to shut down motility completely, was to delete *motB*, one of the last genes involved in flagellar assembly. MotB couples proton motive force to torque generation, and is required to rotate the flagellum. Blair and Berg showed that extrachromosomal expression of MotB in a *motB* mutant strain allowed for restoration and control over motility in an average of 10 min. [7]

Whitesides and coworkers [8] recently demonstrated the ability to direct the trajectory of *E. coli* swarmer cells by controlling microchannel size and composition. This finding presented us with the means to direct our motile bacteria down a one-dimensional course. In addition, the adoption of a microchannel for the bacterial relay race was expected to minimise diffusion of quorum sensing molecules out of the system, so that sender strains would have a maximum effect. Finally, the relative ease of microchannel fabrication offers flexibility in ‘race-track’ layouts.

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2 Description of current work

In order to demonstrate inducible motility, we designed a simple test circuit in which *motB* is placed under control of the *lac* operon promoter (*plac*). We 'BioBricked' the *motB* gene with its endogenous ribosomal binding site (RBS), and this construct (BBa_S03271) is available through the MIT Registry of Standard Biological Parts (http://parts.mit.edu/registry/index.php/Main_Page). We used a *motB*⁻ *E. coli* strain (RP3087) obtained from J.S. Parkinson (University of Utah) for all swarming assays. The immotile phenotype of this strain (relative to a motile control strain) was verified on 0.2% Eiken agar 'swarm' plates [9]. RP3087 cells harboring BioBrick plasmid pSB4A3 (*motB* controlled by *plac*, repA pSC101-derived replication origin, ~10–12 copies per cell) were expected to swarm (become motile) only by induction, upon addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). In contrast, the cells were found to swarm even without induction, indicating that leaky expression from the *lac* promoter was sufficient to confer motility.

In these initial studies, the Lac repressor (LacI) was expressed only from the chromosome. To test whether stronger repression of *motB* could be achieved through higher *lacI* expression, a constitutively-expressed *lacI* was included on the *plac*-controlled *motB* plasmid (Fig. 1). The *lacI* gene was placed downstream of *motB* so that unwanted transcription of *motB* would provide negative feedback. We chose to approach the problem combinatorially: a total of nine constructs contained combinations of promoter and ribosomal binding sites with various strengths placed upstream of *lacI* (Fig. 1). Motility assays were performed, as above, in the presence and absence of IPTG in the swarming media. The results were positive, as we were able to demonstrate induction of motility with IPTG. Only the combination of strongest promoter and medium- or high-strength RBS provided sufficient LacI to repress *motB* before induction (Fig. 2). We thus determined that a mechanism for extremely tight repression of *motB* is necessary in further designs of our receiver cell circuit.

A second circuit for controlling swarming was constructed using the *lux* promoter (*plux*) from *V. fischeri*, which is induced by *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL) [10]. This construct is analogous to the receiver cell input in our final expected design (Fig. 3), removing the 'switch' and placing *motB* as the output, downstream of *plux*. Motility/swarm assays yielded similar results as with *plac*-controlled *motB*: restoration of motility was observed in strain RP3087 harbouring the test construct (pSB4A3) on swarm plates in the absence of HSL inducer. Again, we hypothesised that leaky expression of *motB* from the *lux* promoter produced sufficient quantities of MotB to restore motility in uninduced cells. To verify this leaky expression, the *motB* was replaced with green fluorescent

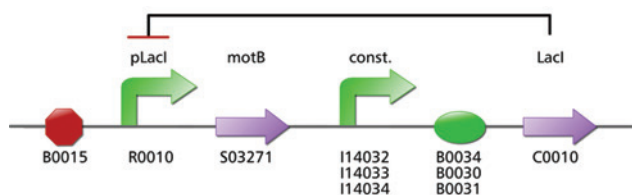


Fig. 1 Design of *motB* repression circuit constitutively expressing *lac* repressor

Numbers below each 'part' refer to the MIT BioBrick Registry. Different strength promoters (I14032, I14033, I14034) and RBS's (B0034, B0030, B0031) were tested combinatorially

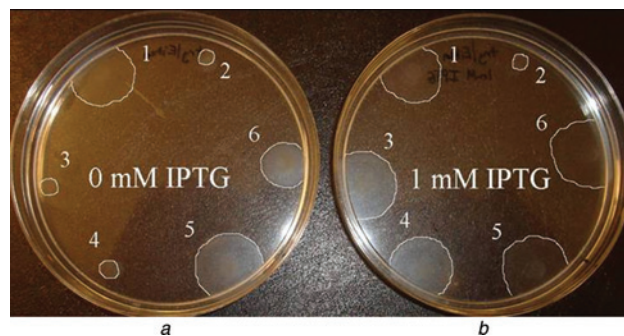


Fig. 2 0.2% Eiken agar plates showing (a) uninduced and (b) induced motility with two of the nine constructs tested

Colony designations: (1) Positive control strain RP437, (2) negative control (*motB*⁻) strain RP3087, (3) construct in Fig. 1 with I14032 and B0034 (highest *lacI* output), (4) construct above with I14032 and B0030, (5) construct above with I14032 and B0031, (6) construct above with I14033 and B0034

All constructs were in low-copy registry plasmid pSB4A3 harboured in strain RP3087

protein (GFP; BBa_E0240) under *plux* control and fluorescence plate reading experiments were performed using various levels of induction with HSL. These experiments confirmed a basal level of GFP expression without induction and a steady increase in GFP expression over 2 h after addition of HSL (data not shown). Difficulties in achieving sufficiently tight control over gene expression when using the *lac* and *lux* promoters have been documented in other iGEM projects.

We reasoned that the strength of the ribosome binding site could be attenuated so that translation from *motB* mRNA derived by leaky expression from promoter *plux* would be insufficient to support motility, while induced cells with much higher *motB* transcript levels could still produce sufficient MotB to swarm. Whereas initial constructs were built from the wild-type *motB* gene with its endogenous RBS (CCGAGGAAGC), we tested five different BioBrick ribosomal binding sites, with efficiencies ranging over two orders of magnitude, in place of the native *motB* RBS and under *plux* control. The *motB* gene without its endogenous RBS (BBa_J09271) was amplified from the chromosome of RP437 (wild-type for motility) using polymerase chain reaction (PCR) and standard BioBrick restriction sites were added to facilitate further

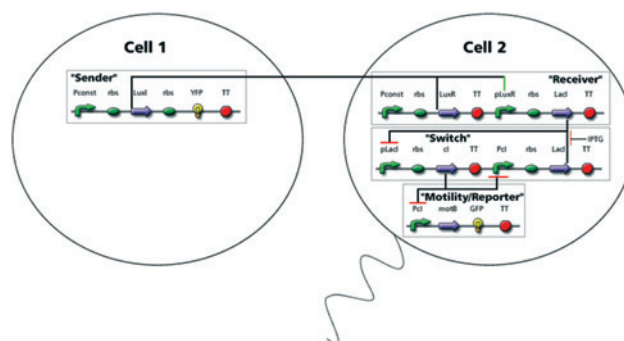


Fig. 3 Proposed genetic circuit

A 'sender' cell (cell 1) expressing *LuxI* will catalyse production of 3OC₆HSL. HSL will diffuse into a second 'receiver' cell (cell 2) and interact with *luxR* which is constitutively expressed in the receiver. This will activate transcription of *lacI*, which is originally turned off. In the 'off' state, *cI* repressor is expressed and represses transcription of *motB*. Activation of *lacI* will flip the 'switch' on and induce transcription of *motB*, thereby restoring motility in the receiver cell. GFP (green fluorescent protein) is co-expressed *motB* as a reporter



Fig. 4 Team photo

cloning. Motility assays were performed as described above to test the various BioBrick RBS constructs. This exercise proved successful, as we were able to identify a combination in which motility was triggered by the inducer HSL with nanomolar sensitivity (not shown). Given this ability to silence motility prior to induction with HSL, our relay race construction was nearing the final stages as we completed the ‘sender’ strain. We also plan to incorporate a bistable toggle switch into this design, from a library of switches provided by Gardner [11]. Alternatives to be explored include options such as riboregulators to silence expression of *motB* [12] or use of *ssrA* protein degradation tags [13] to reduce intracellular concentrations of basally-expressed MotB protein.

Microfluidic channels of polydimethylsiloxane (PDMS) were used in order to direct the otherwise random swarming motion of both the sender cells and the activated receiver cells on an agar floor. Three chief tasks were required for the design of these channels: optimising the thickness and durability of the PDMS channel array, optimising the agar medium on which the cells are grown, and manipulating the surface chemistry of PDMS. The ideal PDMS thickness was determined empirically, and a mold featuring channels of varying widths (10–60 μm) was spin-coated with PDMS at varying speeds.

Low-density Eiken agar was determined to be the most suitable medium. This medium allows the bacteria to swarm in a fluidic environment while supporting them on a solid surface. Several agar densities were tested, and while lower densities supported increased swarming, they were also shown to be less stable when poured into thin layers. The PDMS is naturally hydrophobic, and the project requires the PDMS channels to fill with fluid from the surface of the agar media. A hydrophilic surface was obtained by treating PDMS with O_2 plasma and storage in contact with low-density Eiken agar.

The thickness of the agar itself affects the optical resolution when visualising both bacteria and channels using differential interference contrast microscopy. Higher resolution was obtained by pouring thinner layers of agar; however, the images remained less than satisfactory. A prototype for a slide featuring a small shallow chamber was engineered as a platform for future experiments.

3 Conclusions

Significant progress has been made toward the ability to control and to direct motility in *E. coli*. Through our

experiments, we have demonstrated an ‘off’ state and induction of motility, and we have shown that PDMS microchannels provide a facile means of directing motility. However, we have experienced challenges in cloning and system design, many of which have resulted from the fact that synthetic biology is still a new and evolving science and characterisation of BioBricks for completely predictable design is not yet possible. We hope that continued progress on the relay race project will provide the synthetic biology community with better characterisation of the essential elements of our system and their ability to function in concert.

4 Acknowledgments

The iGEM team (Fig. 4) would like to thank the following additional faculty mentors: Profs S.P. Walker, R. Balasubramanian, W.O. Hancock, M. Tien, D. Farber, B. Nixon, J. Regan, and V. Narayanan. We thank the Penn State Nucleic Acid Facility for supplying PCR and sequencing primers. We also gratefully acknowledge Penn State’s Huck Institutes of the Life Sciences, Eberly College of Science, Materials Research Institute, Office of the Dean of Undergraduate Research, Agricultural and Biological Engineering Department, Chemical Engineering Department, Bioengineering Department, and NSF-funded Center for Nanoscale Science (MRSEC) and Research Experiences for Undergraduates (REU) program for financial support of this work.

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Faculty Mentors: S.P. Walker, Drs R. Balasubramanian, W. Hancock, P. Cirino, M. Tien, D. Farber, P.S. Weiss, B. Nixon, J. Regan, V. Narayanan, and T. Richard.

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SYANAC: SYNthetic biological Automaton for Noughts And Crosses

S. Ayukawa, A. Kobayashi, Y. Nakashima, H. Takagi, S. Hamada, M. Uchiyama, K. Yugi, S. Murata, Y. Sakakibara, M. Hagiya, M. Yamamura and D. Kiga

Abstract: The project of Tokyo Alliance was to construct a bacterial system which plays ‘noughts and crosses’ against a human player. We named it SYANAC, SYNthetic biological Automaton for Noughts And Crosses. An unbeaten strategy of the game could be written in a simple state transition diagram with at most three turns. Based on the diagram, we tried to construct a set of *in vivo* logic gates which determines a move of SYANAC against that of the human player. For the logic gates, inputs are chemicals that regulate protein bindings to corresponding DNA sequences in reporter genes. In order to implement the logic gates efficiently, we standardised the protein-binding sequences and designed a systematic construction method. With the method, it is practical to combine some of these standardised sequences together to construct transcriptional regulatory regions. Since these protein-binding sites are short, we can use chemically synthesised DNA as a part. A regulatable gene was constructed by insertion of a $-35/-10$ promoter part and LacI-binding-site parts into a promoterless reporter plasmid which can also accommodate canonical Biobricks. This new method, thus, will allow us to construct a set of logic gates by combining standardised protein-binding parts and Biobricks and to realise the game.

1 About team Tokyo Alliance

The Tokyo Alliance team consisted of four undergraduate students and one graduate student from three universities in Tokyo, Japan. Shotaro Ayukawa and Shogo Hamada belong to Tokyo Institute of Technology; Akio Kobayashi belongs to University of Tokyo; and Yusaku Nakashima and Hidemasa Takagi belong to Keio University. All five students gathered in Kiga Laboratory in Tokyo Institute of Technology on the summer of 2006 and worked for their iGEM 2006 project.

2 Aims of the project

Noughts and crosses, also called tic-tac-toe, is a well-known board game (Fig. 1), played by two people each of whom takes turns to mark the space in a 3×3 grid. The player who completes a horizontal, a vertical or a diagonal row wins the game.

The game has already been implemented *in silico* and *in vitro*. The game implemented *in silico* was played

against a human player by EDSAC in 1949. In 2003, this game was also implemented *in vitro* with deoxyribozymes and it was played by MAYA [1]. It had a strategy described by an automaton to be unbeaten.

Then our project for iGEM2006 was to implement another unbeatable automaton for noughts and crosses *in vivo*: SYNthetic biological Automaton for Noughts and Crosses, or SYANAC. Instead of hybridisation between the deoxyribozyme and input oligo DNA, we decided to use the binding, regulated input chemicals, between transcriptional regulatory by proteins and their corresponding DNA sequence. Noughts and crosses on the game board are shown by the green and red fluorescence of *E. coli* transformants, each of which responds to the human-player move (Fig. 2).

In this project, there are two significant points for the development of synthetic biology. Firstly, we attempted to increase the number of available inducers. One of the problems in synthetic biology is that there are only a few transcriptional inducers to control a genetic circuit. A massive integrated biological circuit will require a variety of inducers which can regulate the genetic circuit. Secondly, through this project, we proposed a systematic design method for the assembly of standardised protein-binding-site parts. Construction by a combination of standardised parts allows efficient implementation of a regulatory region which can accommodate multiple regulatory proteins. Therefore constructing SYANAC has an important meaning for the development of synthetic biology.

3 Description of work

3.1 Transition diagram of the automaton for noughts and crosses

What we had to do first was to construct a state transition diagram for SYANAC based on our strategy of noughts

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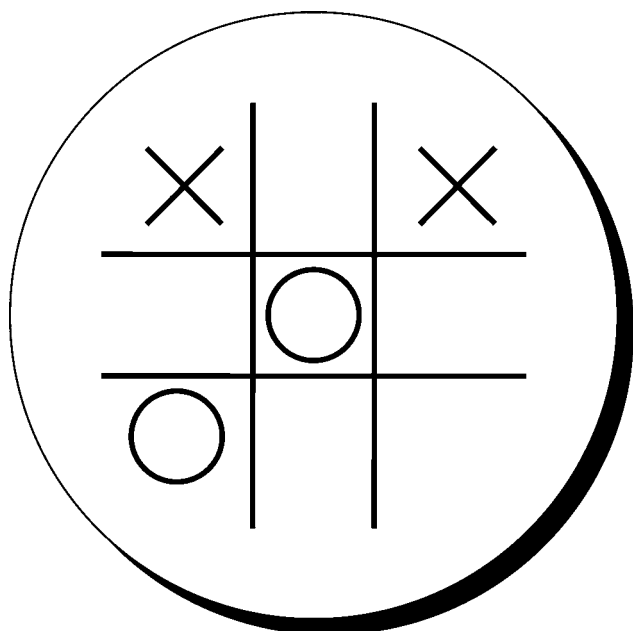


Fig. 1 Image of noughts and crosses

and crosses. Calculating all of the SYANAC's move, we designed a state transition diagram (Fig. 3) of a small-sized automaton based on an unbeatable strategy since it is difficult to implement all possible flows of noughts and crosses.

For the design, we found a way to simplify and reduce the size of this tic-tac-toe problem by using symmetry of the board and modification of a previous strategy of automaton. In SYANAC, there are two rules for reducing the number of inputs based on symmetry. These rules were also used in MAYA. The first rule is that the game is always started by SYANAC and it shows optimal move to Sq. #5, the central square of the grid. The second rule is that the human player marks either Sq. #1 or Sq. #4 in his first turn. Because of the symmetry of the board, Sq. #1 is equivalent to the other corner squares, #3, #7, and #9. So does Sq. #4. Sq. #4 is equivalent to the other non-corner squares in terms of symmetry. Therefore this simplification does not reduce the possible choices of a human player.

In order to reduce the number of inputs, we also improved a strategy of SYANAC from that of MAYA which had the state transition diagram with eight inputs [1].

Because of the symmetry, the first move of human player is restricted to either Sq. #1 or Sq. #4 as described above.

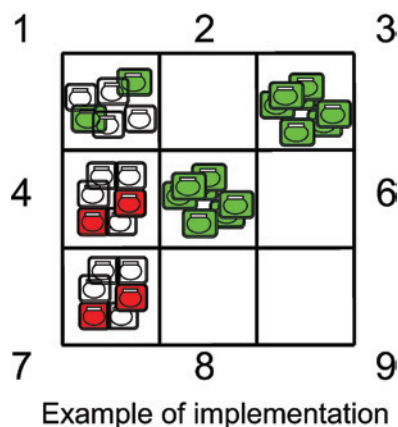


Fig. 2 In vivo noughts and crosses

Noughts and crosses on the game board are shown by the green and red fluorescence of *E. coli* transformants

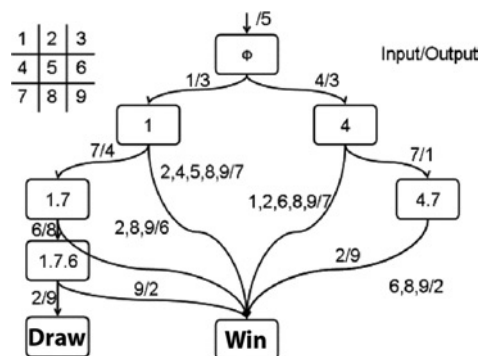


Fig. 3 State transition diagram

The move of SYANAC is determined by a history of human moves. Each number in the diagram indicates a square number of the grid. The number on the left side of '/' represents an input from human player. The number on the right side of '/' represents output by SYANAC as a response to an input. For example, '2, 8, 9/6' means that when human player marks either Sq. #2, #8, or #9, SYANAC will mark Sq. #6. The numbers in open rectangles indicate the history of human moves. For example, 4, 7 in an open rectangle indicate that human player already has marked Sq. #4 and #7

We found that the second move of SYANAC on Sq. #3 leads one of the perfect strategies independent of the initial human move. As a result, we reduced the number of squares with the possibility of human input from eight to seven, and made the simplified state transition diagram.

According to the state transition diagram, for the completion of SYANAC, we need three classes of logic gates: 13 YES gates, 11 AND gates, and 10 ANDAND gates. The YES gate is a logic gate which returns an output when one input comes. In an AND gate, coexistence of two different inputs causes an output. In the ANDAND gate, coexistence of three different inputs causes an output. Based on the transition diagram, we allocated logic gates in an appropriate square of 3x3 grid. Note that one or more number of logic gates are allocated into each square (Fig. 4).

Each logic gate is introduced into *E. coli* in the form of a plasmid. This means that each square contains one or more kinds of *E. coli* transformants. Each *E. coli* transformant produces fluorescence according to the flow of the game to the flow of the game indicated by chemicals.

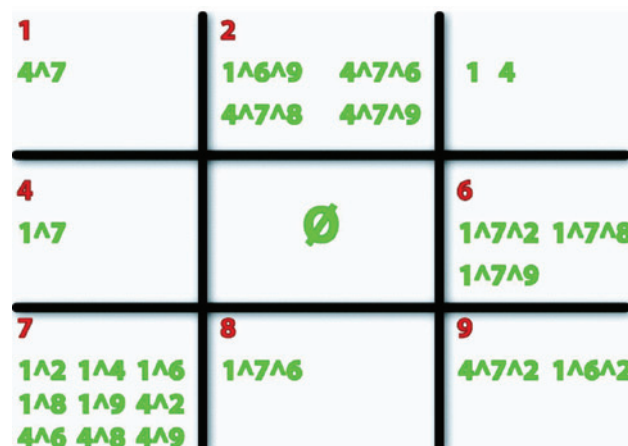


Fig. 4 Assignment of logic gates

Assignment of logic gates is dependent on the transition diagram shown in Fig. 3. Each square contains several number of logic gates; for example, Sq. #1 contains two logic gates; a YES gate expressing RFP (shown in italics) stimulated by the input chemical I#1 and an AND gate expressing GFP (shown in bold) stimulated by coexistence of input chemical #4 and #7

Table 1: Regulatory protein and their inducer chemicals; candidates of regulatory proteins and their inducers are listed. The binding of a regulatory protein to a DNA is controlled by its inducer chemical [2–11]

Regulatory protein	Inducer chemical	Reference
LacI	IPTG	[2]
TetR	aTc	[3]
FadR	oleate	[4]
RbsR	D- ribose	[5]
AraC	arabinose	[6]
LuxR	AHL	[7]
MelR	melibiose	[8]
AgaR	N- acethylgalactosamine	[9]
BetI	Choline	[10]
EmrR	DNP, salicilate	[11]

3.2 Design concept of a logic gate plasmid

The logic gate used in SYANAC was designed in the form of an engineered regulatory region of a reporter gene. The regulatory region includes a $-35/-10$ sequence, a ribosome-binding site, a GFP reporter coding sequence and one or more protein-binding sites. Specific proteins bind to particular protein-binding sites dependent on input chemicals which indicate the history of human moves and control the logic gate. Thus, designing the protein-binding site is the most consequential part in controlling the GFP expression. In the case of the YES Gate, one inducer binds to a regulatory protein and subsequently stimulates GFP expression. In the AND gate, the combination of two different chemicals regulates the expression. In the case of the ANDAND gate, the combination of three different chemicals regulates the expression.

3.3 Candidates of inducible regulators

In order to make a complete set of logic gates for the game, at least seven different inducers were required. Based on references, we picked up ten regulatory proteins shown in Table 1 as candidate parts of logic gates [2–11].

Every part has to work as a YES gate which regulates gene expression dependent on a corresponding inducer chemical. For example, IPTG, an inducer of LacI, stimulates gene expression. In the absence of IPTG, LacI binding to a regulatory region represses the expression of GFP. When IPTG is added, LacI is released from the regulatory region and GFP expression occurs. In this way, SYANAC can return GFP expression as output dependent on several combinations of inducer chemicals.

3.4 DNA construction method

Design of various types of logic gates with several inputs requires allocation of multiple protein-binding sites in a regulatory region. Lutz and Bujard put AraC- and LacI-binding sequences in a 150 bp regulatory region and constructed one AND gate-like regulation [12]. However, there was no modularity between the binding sites and thus no expandability in their method. Their method is therefore not efficient for implementation of three different classes of logic gates: YES gate, AND gate and ANDAND gate. Considering the efficiency of Biobrick construction strategy, combinatorial synthesis using several kinds of protein-binding sites is appropriate for implementation of these logic gates. For this reason, we divided a regulatory

region of a reporter gene on a plasmid into three areas: upstream, midstream, and downstream (Fig. 5).

Division of the regulatory region allows us to construct many logic gates systematically. Sometimes after validation of $-35/-10$ region, the new insertion of protein-binding sequence between this region and RBS is required. The current Biobrick method, however, does not allow such an insertion. Based on the Biobrick method, we extended this idea into the modification of the promoter region using an additional set of prefix and suffix between the canonical set. Following the prefix and suffix idea, this new set allows sequential insertions of protein-binding sites. With this method, a protein-binding sequence containing $-35/-10$ sequence or a midstream part can be inserted between Sal I and Bam HI sites. Upstream parts can also be inserted using the same method in front of midstream parts. Downstream parts can be inserted between Bgl II and Mlu I sites.

Another strong point of this strategy is that we can use chemically synthesised oligo DNA as a part since a protein-binding part is short. With this simple parts preparation and construction method, any number of protein-binding sequences can be inserted into a regulatory region very efficiently.

3.5 Result

With the systematic construction method, we confirmed that insertion of and regulation by some parts worked as designed. The YES gate containing a constitutive active $-35/-10$ promoter part and LacI-binding sites in its downstream area expressed GFP when IPTG was added. Fig. 6a shows the change of GFP fluorescence regulated by IPTG. After 6 h incubation, GFP fluorescence of the culture with IPTG became 1.5 times greater than that without IPTG.

Canonical iGEM parts are also able to be inserted into our plasmid. We inserted a Biobrick BBa I0500 which contained an AraC-binding site and a weak promoter into an upstream area of our new prefix site of a promoterless plasmid. The resultant plasmid worked as a YES gate and

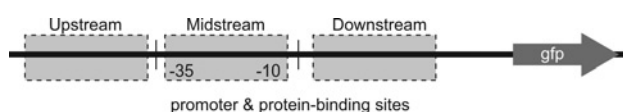


Fig. 5 Division of the regulatory region

The regulatory region of a reporter gene was divided into three areas: upstream, midstream and downstream. The division allowed insertion of several protein-binding sites for construction of logic gates

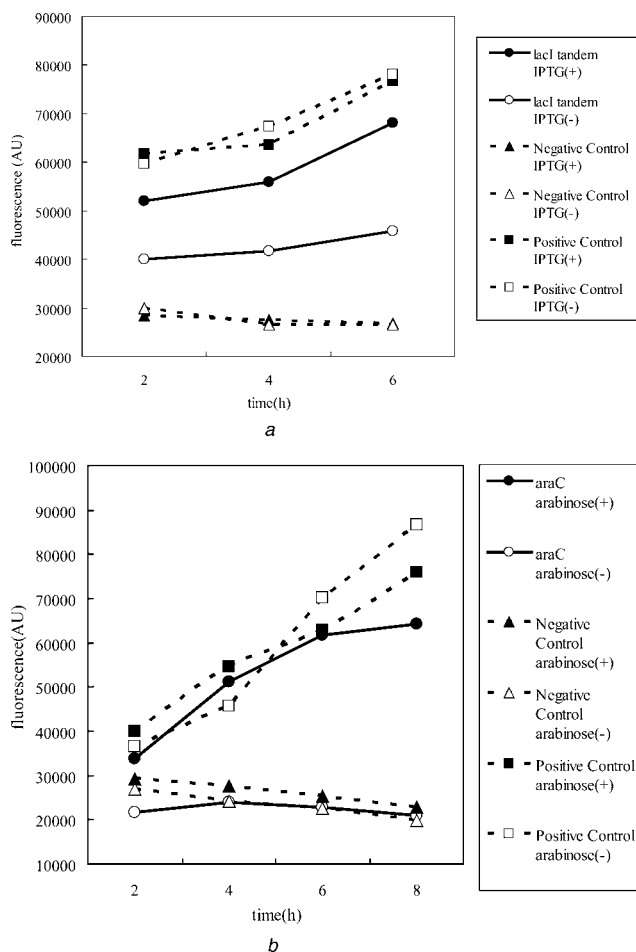


Fig. 6 Change of GFP fluorescence regulated by an input chemical

The fluorescence of *E. coli* transformants incubated in the presence or absence of input chemicals was monitored. Input chemicals had no effect on the fluorescence product by *E. coli* transformants which constitutively express GFP (positive control) and which have promoterless plasmids (negative control). Input chemicals increased the fluorescence product by *E. coli* which were introduced YES gates

a Change of GFP fluorescence regulated by IPTG

b Change of GFP fluorescence regulated by arabinose

expressed GFP when arabinose was added, as shown in Fig. 6b. After 6 h incubation, GFP fluorescence of the culture with arabinose became three times greater than that without arabinose.

4 Conclusion

In this work, we showed a systematic construction strategy which allows efficient building of logic-gate plasmids with multiple binding sites for regulatory proteins. These sites were classified into three types based on their insertion area: upstream, midstream and downstream. The AND gate can be implemented by a combination of binding-sites, each of which works alone as a YES gate and classified in different types each other, such as the combination of upstream AraC-binding and downstream LacI-binding parts. In addition, the combination of LuxR- and LacI-binding sites will also work as an AND gate because binding sites classified in the same type, such as upstream AraC- and LuxR-binding parts, are compatible. With the same idea, an ANDAND gate can be designed by the additional introduction of a midstream part such as an EmrR-binding site. The ANDAND gate will respond to combination of three inducers, arabinose, salicylate and IPTG.

Since we already picked up the candidates for regulator parts and established a reliable construction method, it will be a straightforward procedure to construct 30 plasmids in one month even for undergraduate students. Because implementation of SYANAC requires only 11 AND gates and 10 ANDAND gates, it may not be so hard to complete a set of logic gate for SYANAC.

Our systematic construction method and standardised parts can be applied to advanced engineering of synthetic biology. The simple construction method we established through the implementation of SYANAC allows very efficient construction of logic gates regulated by multiple inducers. Such logic gates must be required to regulate complicated systems implemented inside living cells. Therefore our study will contribute to development of engineering skills in synthetic biology.

5 Acknowledgments

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Engineering a molecular predation oscillator

R.I. Kitney, P.S. Freemont and V. Rouilly

Abstract: The paper addresses the problem of designing and building a stable molecular based oscillator which can be controlled in terms of both amplitude and frequency. A study of previous oscillators of this type showed that they are inherently unstable. To overcome this problem a design was chosen which is based on Lotka–Volterra dynamics. An important aspect of the work was the use of what we term the Engineering Cycle; that is, the cycle of system specification, design, modelling, implementation, and testing and validation. The Lotka–Volterra dynamic, in the context of a predation oscillator, amounts to a predator–prey approach. This is the basis of the oscillator design. The oscillator was designed and detailed modelling undertaken to establish the modes of the dynamic; how it could be tuned for stability; and how to control its amplitude and frequency. The biological implementation of the design was undertaken using a number of BioBricks from the MIT registry (http://parts.mit.edu/registry/index.php/Main_Page), together with a number of parts which we designed and built.

1 Introduction

The paper describes the design and implementation of the molecular predation oscillator which formed the Imperial College submission to the iGEM competition in 2006. The team came second overall in the competition. The approach which was used was the traditional engineering approach for building a stable and flexible oscillator (in this case molecularly based). As will be shown in sub-sections of this paper, this approach involved a step-by-step development cycle comprising specifications, design, modelling, implementation and testing/validation. The full documentation for the project can be found on our OpenWetWare site: <http://openwetware.org/wiki/IGEM:IMPERIAL/2006>

2 Specifications

As with standard engineering oscillators (mechanical, electronic, fluidic etc.) it was necessary to define basic specifications. The key specifications are:

- stable oscillations for more than ten periods
- high signal-to-noise ratio
- controllable frequency and amplitude
- modular design for easy connectivity
- full documentation for quality control

Having defined the specifications, the next task was to study a range of approaches for implementing the oscillator as a biological system. This led to a decision to develop an oscillator design based on cell population dynamics. The mathematical basis for this was the Lotka–Volterra

predation model. Its biological implementation was based on the quorum-sensing system found in *Vibrio fischeri*. Fig. 1, below, shows the basic dynamic of the oscillator design. This amounts to a predator–prey approach to the left-hand side of the diagram (i.e. the prey generator) representing the dynamic generation of the prey molecule population and the right-hand side of the diagram representing the dynamic generation of the predator molecules.

A key aim of the iGEM competition is to use, where possible, BioBricks which are available from the MIT Registry (parts.mit.edu). The central row in Fig. 1 shows the BioBricks which were used from the registry. These were added to new constructions which were developed during the project (and indeed in a number of cases became new BioBricks for the registry). Specifically, quorum sensing/quenching BioBricks from the registry were used in the design. The design of the oscillator was reduced to a system of two cell types in order to produce greater flexibility.

3 Modelling

A very important aspect of the design cycle was the development of a detailed computer model of the predator–prey system. This dynamical model was derived from a theoretical analysis. One of the key aspects of the modelling was to determine the conditions for stable oscillations which were controllable in terms of frequency, amplitude and profile. Fig. 2 shows details of the equations. Referring to the figure it can be seen that there are a number of parameters which can be controlled: a , b , c (population dependent); a_0 , b_0 , c_0 (constants); and d_1 , d_2 , e (washout related).

Fig. 3 illustrates the functional and intermediate parts which were used in the oscillator. It can be seen from the figure that some of the parts were built, sequenced, tested, characterised and documented, while others were simply built and sequenced. It should also be noted from the diagram that parts were defined not only in

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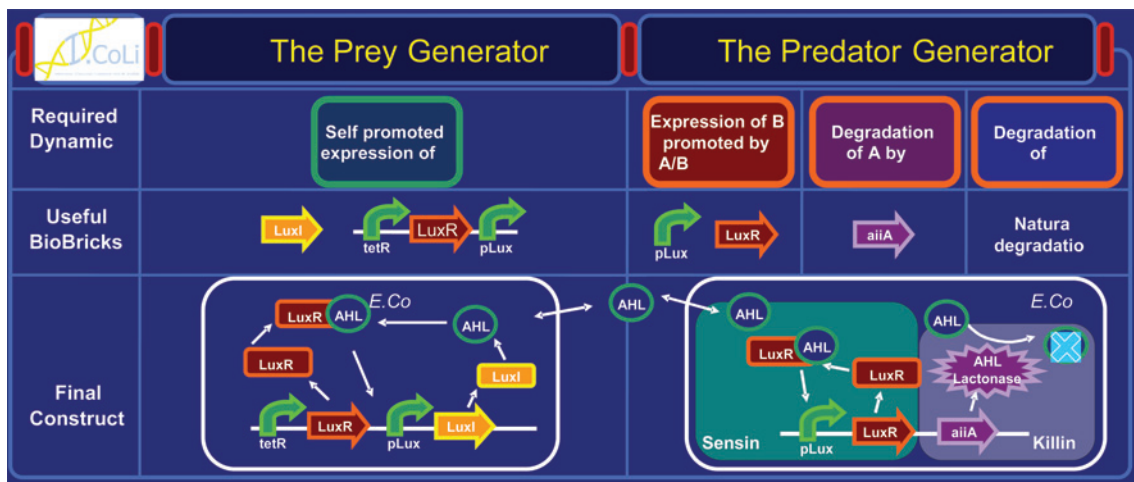


Fig. 1 Biological design of the predator generator

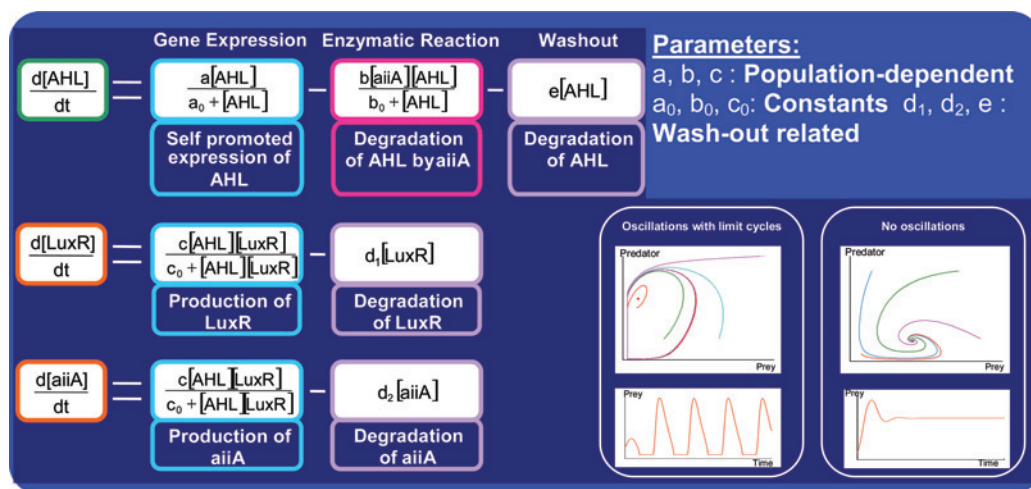


Fig. 2 Modelling of the full oscillator system, including limit cycle studies

Functional Parts		Built	Sequenced	Tested	Characterized	Documented
Final Prey J37015		✓	✓	✓	✓	✓
Sensing Prey T9002				✓		✓
Sensing Predator J37016		✓	✓	✓	✓	✓
Cre/Lox J37027		✓	✓	✓		✓
Intermediate Parts		Built	Sequenced	Built Sequenced		
J37033		✓	✓	✓	✓	✓
J37034		✓	✓	✓	✓	✓
J37019		✓	✓	✓	✓	✓
J37032		✓	✓	✓	✓	✓
J37023		✓	✓	✓	✓	✓

Fig. 3 Contributions to the MIT Registry as a result of the project: functional and intermediate parts

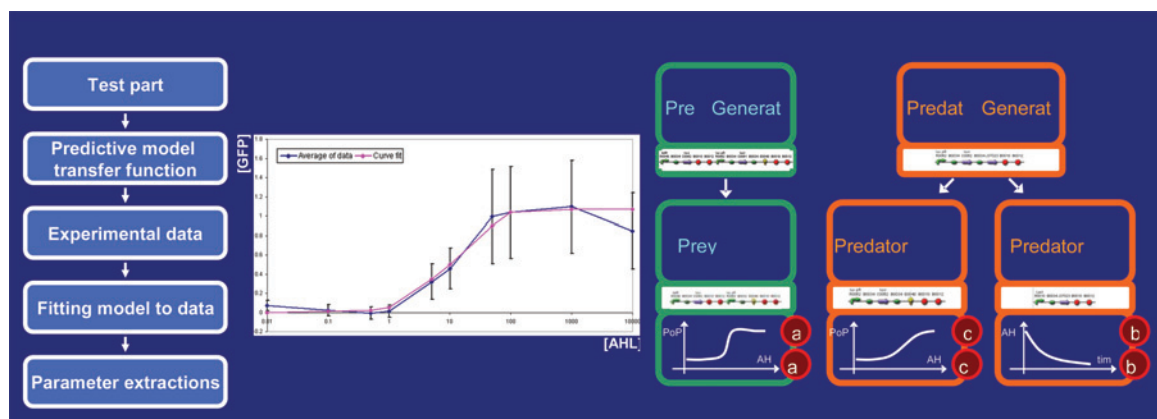


Fig. 4 Characterisation of predator sensing prediction of the model transfer function; fitting the experimental data; and extracting parameters

terms of a text title but in terms of an alphanumeric part (e.g. J37015).

4 Testing and validation

The initial step was to define testing protocols to satisfy component specifications. This was followed by the analysis of experimental data (both in terms of that acquired in the wet lab and from the model). This allowed the characterisation of different test constructs. The specific procedure for testing and validation comprised testing the part; predicting the model transfer function for the part; acquiring experimental data; fitting the model to the acquired data; and extracting the appropriate parameters. Fig. 4 illustrates this procedure. It should be noted from the figure that, for example, a plot of GFP against AHL shows wide variation for individual runs (but an expected characteristic, in terms of the model prediction). This is important because it shows that one of the features of biologically based oscillators is their high variability, high noise and so on. This is interesting because this was the situation when the original electronically based oscillators were built. Hence, it is anticipated that one of the key areas

of development for biologically based oscillators will be (as with the original electronic versions) to significantly variability and noise.

5 Conclusion

Within the project a complete dynamical model was derived which describes the main biochemical reactions which characterise and drive the molecular oscillator. This was coupled to a full theoretical analysis and detailed computer simulation. On these bases, we were able to validate the design in relation to the original specifications. This resulted in the successful construction and characterisation of functional parts which are the building blocks for the planned oscillator.

6 Acknowledgments

R.I. Kitney, P.S. Freemont and V. Rouilly are the authors of the paper but carried out the project with an undergraduate project team. Their names are Christian Sander, Farah Vohra, Jiongjun Bai, John Sy, John Chattaway, Jonathan Wells and Tom Hinson.

A microbial biosensor device for iron detection under UV irradiation

A. Quintero, S. García, C. Guevara, C. Rincón, C. Ospina, P. Guevara and R. Cuero

Abstract: Biosensors are useful molecular and/or cellular tools that allow detection of the presence of different metals including iron ($\text{Fe}^{\text{II/III}}$) and other compounds, even at detection levels beyond the limits of conventional methods. Bacterial cells were transformed with pSB1A3 vector containing promoters PI and PII from *Acidithiobacillus ferrooxidans* rus operon and a LacI regulated device coding a monomeric red fluorescent protein (mRFP1). An MIT BioBrick containing different parts was used to assemble the machine. The designed device was standardised for its specific detection using iron ions and/or IPTG as inputs and mRFP1 as output. Transformed cells were grown under the presence of UV radiation (360 nm) or fluorescent light, and different Fe^{II} concentrations (0, 1, 50, 100 ppm). The response of the biosensor was measured by the expression of reporter protein, DNA fluorescence and/or concentration, bacterial growth, and redox potential (mV/pH) of the bacterial culture. The machine was correctly assembled and transformed in *E. coli*, which was observed through agarose gel electrophoresis of the plasmid and the total DNA. A ~ 3.506 kbp band was obtained in each case. The viability of the machine was also confirmed by the detection according to the iron concentration, and fluorescence of the reporter protein. The aim of the project was to use synthetic biology in order to develop a microbial biosensor machine assembling new protein promoter sequences for iron uptake, and some standard parts (BBa_J04450) from MIT BioBricks made in *Escherichia coli* DH5 α strain (provided by the International Center for Tropical Agriculture, Colombia) to detect ion metals such as Fe^{II} , under a UV light environment.

1 Introduction

A biosensor is an analytical device that couples microorganisms with a transducer to enable rapid, accurate and sensitive detection of target analytes. They have applications in various fields including environmental monitoring, medicine, food processing and safety [1]. Microbial molecules such as RNA can be used as highly sensitive markers for the detection of toxic metals (e.g. lead) and radionuclides (e.g. uranium) [2].

High iron concentrations are associated with some extreme environments found on Earth where only a few microorganisms can survive. The presence of iron has also been verified in extraterrestrial environments such as Mars. The assembly of a biological system able to respond to extreme conditions (and additionally under UV presence) that resemble conditions on Mars, will help us to understand better the transition from inorganic molecules to organic compounds (biogenesis) on Earth and what life really is as a physical, chemical and biological system [3]. Earth and Mars are part of the inner planets of the Solar System, and are made of rocks (silicates) with iron-rich cores [4]. Reports from NASA's exploration of Mars have demonstrated the presence of large amounts of UV radiation

in its atmosphere [4]. The presence of UV radiation and a high iron concentration could result in a photon-fenton interaction, thus mediating oxidation-reduction processes in iron. Thus, since detection of any metal depends on its state of oxidation-reduction, the use of UV radiation can mediate the detection of the metal (i.e. iron).

2 Description of the research work

2.1 Plasmid isolation, preparation of competent cells and cell transformation

In order to achieve the denoted objective, a genetically engineered machine was assembled for detection of iron at different concentrations under UV irradiation. Different gene sequences including protein promoters for iron uptake, along with standard parts from the MIT BioBrick Registry were constructed and assembled in vector pSB1A3 (Fig. 1) into a chassis (*E. coli* Dh5 α). Sequences for iron uptake were synthesised (Genemed Synthesis Inc, San Francisco). Promoters PI and PII from the autotroph bacteria *Acidithiobacillus ferrooxidans* rus operon, which encodes the protein rusticyanin, considered an important component in iron oxidation [5], were used. Competent cells were prepared following the OpenWetWare MIT-Harvard-Berkeley protocol. Vector BBa_J04450 was selected from iGEM DNA repository plate 2 and transformed in *Escherichia coli* DH5 α strain by electroporation and chemical transformation [6]. DNA extraction was performed from single bacterial colonies using a Wizard Plus Minipreps DNA Extraction and Purification System (Promega), in order to amplify the genetic material, and this was followed by electrophoresis.

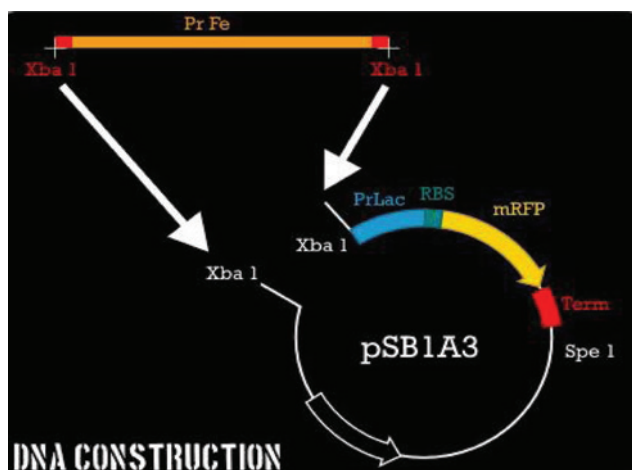


Fig. 1 Constructed biosensor device for iron detection under UV irradiation

2.2 Enzyme digestion and ligation

Enzyme digestion with XbaI and ligation with T4 DNA ligase were done according to standard processes [7]. *Escherichia coli* bacterial cells were transformed and subjected to different concentrations of iron (0, 1, 5, 50 and 100 ppm) in LB liquid medium or on LB agar, under presence of UV radiation (360 nm) or fluorescence light. Bacterial cells were incubated under an alternate regime:

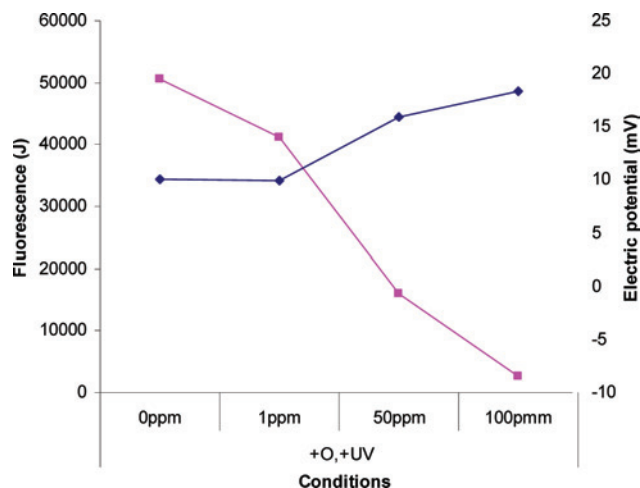


Fig. 3 DNA fluorescence (dark line) and electrical potential (mV) (light line) vs. iron concentration

10 h of continuous fluorescent light. Some bacterial cells were then exposed to UV radiation for 8 h, while other samples continued under fluorescence light for an additional 8 h. The bacterial cells were incubated at room temperature.

Different parameters were measured and related, protein expression, which was determined by visual observation (Fig. 2), DNA quantification was determined by sibergreen dye in a microplate reader (Tecan Genius, USA), bacterial

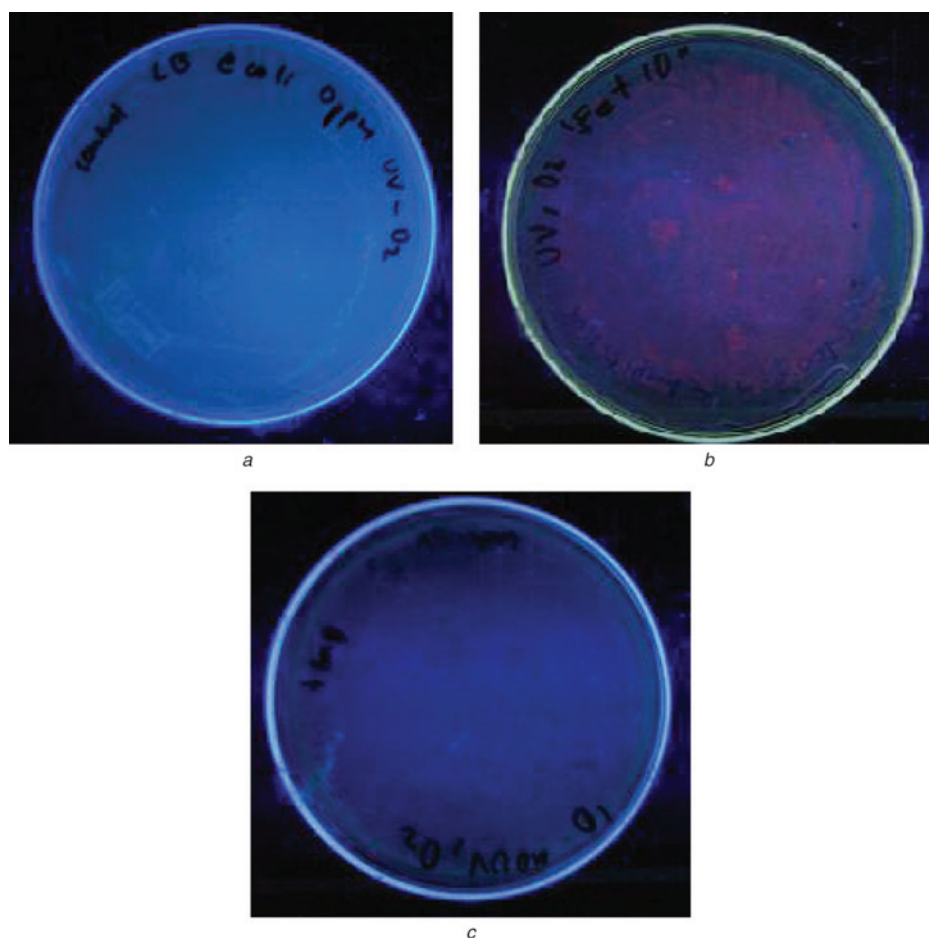


Fig. 2 mRFP1 expression on agar medium with different concentrations of iron and exposed to UV light for 8 h

- a 0 ppm (control)
- b 100 ppm
- c 100 ppm without UV light (control)

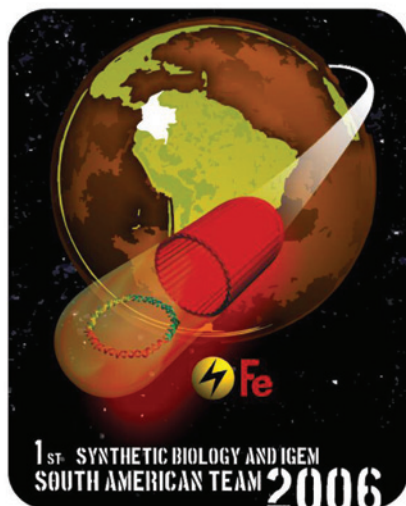


Fig. 4 The Colombian iGEM 2006 team and logo (designed by P. Guevara)

growth on agar (CFU) and redox potential (mV), which was related to DNA concentration (Fig. 3). The biodetection was established by correlating expression of the protein promoter and/or DNA concentration with the concentration of iron (Fig. 3).

3 Conclusions

Different parts were successfully assembled: promoters PI and PII, promoter LacI, ribosomal binding site, coding region of mRFP1, and two transcriptional terminators B0010 and B0012 (Fig. 1).

The constructed machine was functional. There was a direct correlation between detection and iron concentration, as well as between detection and protein expression in relation to iron concentration, and between electrical potential and DNA fluorescence in relation to iron concentration (Fig. 3).

4 Acknowledgments

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Vanillin cell sensor

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Abstract: Our project for iGEM 2006 consisted of designing a cellular vanillin biosensor. We used an EnvZ–*E. coli* strain as a chassis, and constructed two different devices: a sensor and an actuator, assembled using OmpR-P as a standardised mediator. The sensor device contained a computationally designed vanillin receptor and a synthetic two-component signal transduction protein (Trz). The receptor protein was based on a ribose-binding protein as scaffold. The Trz was built by fusion of the periplasmic and transmembrane domains of a Trg protein with an EnvZ kinase domain. When the receptor complex binds Trg, an allosteric motion is propagated to the cytoplasmic EnvZ kinase domain, resulting in autophosphorylation and subsequent phosphate transfer to the OmpR transcription factor, which finally induces transcription of the ompC promoter. As actuator, we used a synthetic transcriptional circuit, which implements an OmpR-P band detector having GFP and RFP as an output. We designed this circuit using a synthetic promoter working as an AND gate, which is synergistically activated by cI and CRP. Our constructed Trg-EnvZ fusion and AND promoter will be very useful to future synthetic biology projects.

1 Aims of the project

The main goal of this project was to introduce students to synthetic biology through the iGEM competition [1], while trying to do an interesting project that could generate parts to be reused by many future projects in synthetic biology. We chose to design a cellular biosensor having vanillin as an input and the expression of two reporters as output. To perform this, we constructed two devices: a sensor (based on a receptor and a two-component signal transducer) and an actuator (which received the intermediate signal and expressed the reporters). We used a phosphorylation mechanism from EnvZ as transmembrane pathway using OmpR-P as a standardised mediator (Fig. 1). To avoid interference with the natural pathways and have a better control of the kinase/phosphatase domain, we chose an EnvZ–*E. coli* strain as a chassis.

The receptor domain of the sensor was inspired on the work by the group of Dr Hellinga, in which a sensor of

TNT and other molecules using a mutated a ribose binding protein (RBP) were designed [2]. We have used computational protein design [3] to design RBP for ligand affinity towards vanillin, making *E. coli* able to taste flavour (see Fig. 2). The synthetic transducer was constructed by fusing the periplasmic and transmembrane domains of a Trg protein with that of an EnvZ kinase [4]. When the vanillin-receptor complex binds Trg, an allosteric motion is propagated to the cytoplasmic EnvZ kinase resulting in autophosphorylation, followed by phosphate transfer to the OmpR transcription factor (OmpR-P), which is then able to induce transcription of the ompC promoter. The actuator device had OmpR-P as input and GFP (part BBa_E0040) and RFP (part BBa_E1010) as output (see Fig. 1).

To express the reporters according to the vanillin concentration, we used a synthetic genetic network implementing a band detector. At intermediate OmpR-P concentration levels RFP is expressed, whereas GFP is only expressed at high concentration levels. Hence, we obtained a graded response according to the vanillin concentration (see Fig. 3). This synthetic promoter is synergistically activated by two transcription factors cI and CRP (implementing an AND logic gate) [5].

We also envisaged an alternative design plan that avoided using an AND promoter. It consisted of using an alternative circuit, relying only on promoters regulated by a single transcription factor (see Fig. 4). We would keep the use of the λ -cI and TetR in order to reuse part of the original circuit. In fact, this design took full advantage of existing composite parts from the Registry of Standard Biological Parts from MIT [6] and did not require a newly designed promoter.

Using two types of ompC promoters (wild-type and designed) with different activation thresholds forced us to characterise the corresponding existing promoters before assembling our system. From the simulations of our designed circuit, we found that our TetR inverter required a strong repression level, as a basal expression of cI could produce undesired results.

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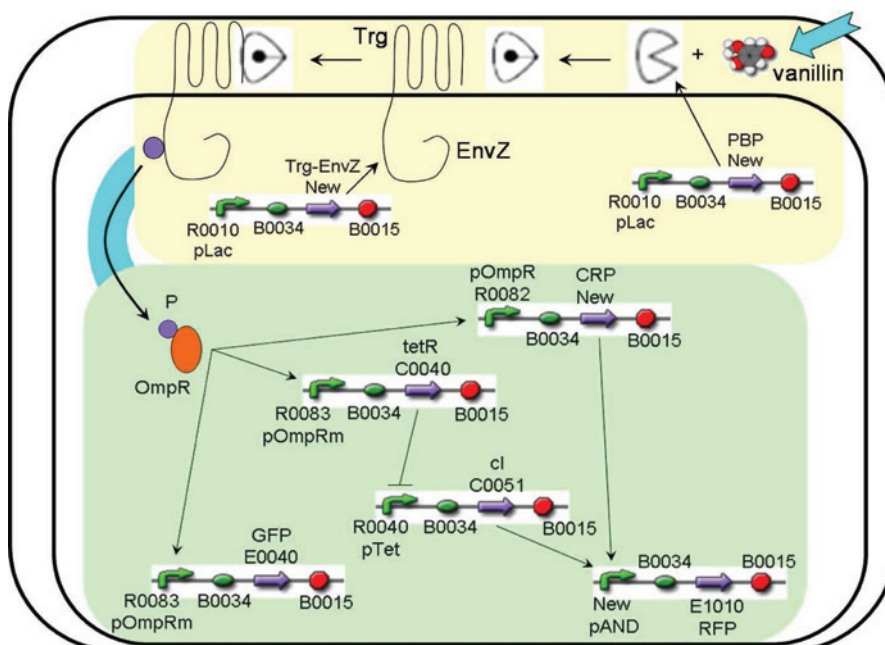


Fig. 1 Biological scheme of our designed system using parts [7]; the yellow set shows the sensor device and the green one the actuator

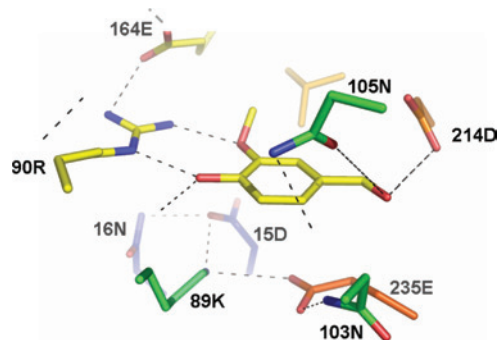


Fig. 2 Detail of the active site of the computationally designed vanillin-binding protein

Dashed lines denote H-bonds relevant for binding of vanillin or complex stability

2 Description of the work

2.1 Parts design and construction

We designed 24 parts and constructed 6, which are now included in the Registry [6]. We designed a synthetic periplasmic binding protein that docks a vanillin molecule (submitted as part BBa_J58105) using a computational procedure [3] and RBP (pdb code 2DRI) as a scaffold (see Fig. 2). To design our AND-type promoter (submitted as part BBa_J58100), we took the λ -Prm promoter from the Registry (part BBa_I12007) as a starting point. The centred position of the cI operator was fixed to 42 and for the CRP to 93.5 [6] (see Fig. 5). Unfortunately the CRP consensus binding site contained an XbaI site, which is used to perform the standard assemblies [7].

In addition, we constructed a reporter by assembling our AND promoter (submitted as part BBa_J58101) with the RFP generator including an RBS and a terminator. In this way, we obtained a single genetic device

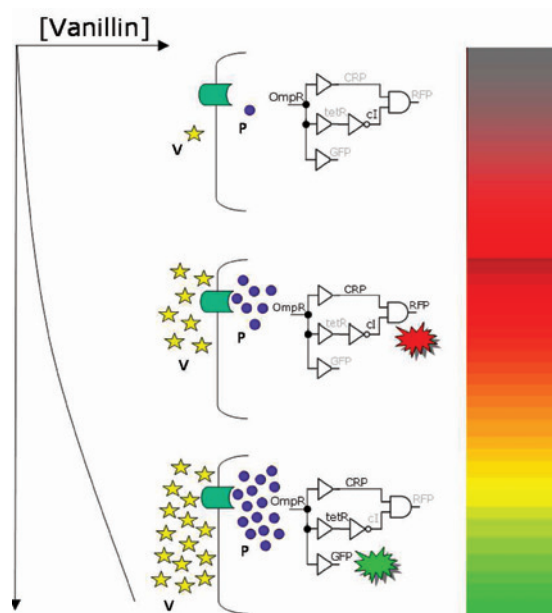


Fig. 3 Mechanism of the system

Plot of the reporters according to the vanillin concentration; in addition we can see the analogous electronic circuit of our actuator device

that produces RFP when enough levels of cI and CRP are simultaneously reached. We also constructed another single device (submitted as part BBa_J58102) that expresses GFP when the OmpR transcription factor is phosphorylated. It was built by assembling the ompC promoter (part BBa_R0082) with the GFP generator. Furthermore, we implemented an additional GFP generator (submitted as part BBa_J58103) with a mutation in the promoter region (part BBa_R0083). This promoter contains a single operator in its sequence, whereas in the natural one three operators are present. Finally, the DNA sequence of our chimeric Trz chemoreceptor (submitted as part BBa_J58104) was

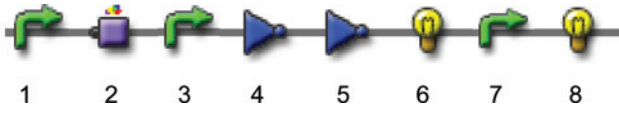


Fig. 4 Alternative design for the actuator device (band detector) using available parts from the Registry [7]

- 1 Mutated ompC promoter (part BBa_R0083)
- 2 TetR generator (part BBa_P0440)
- 3 ompC promoter (part BBa_R0082)
- 4 cI inverter (part BBa_Q04510)
- 5 TetR inverter (part BBa_Q04400)
- 6 RFP reporter (part BBa_I13507)
- 7 same part as 3
- 8 GFP reporter (BBa_I13504)

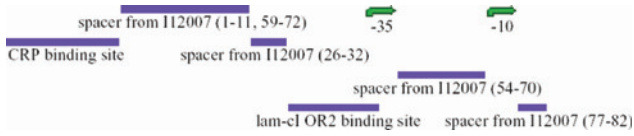


Fig. 5 Scheme of the different fragments used to design the DNA sequence for our AND promoter, synergistically activated by cI and CRP [6], (see part BBa_J58100 in the Registry)

The number represents the part number (without the BBa_ prefix), the nucleotide range is shown in brackets

obtained by joining the transmembrane domain (as well as a short cytoplasmic segment) of Trg and the kinase/phosphatase cytoplasmic domain of EnvZ at the NdeI restriction site [4].

2.2 Devices: simulations and characterisation

Our system used two reporters as output: RFP and GFP. We used an incoherent genetic circuit [9] to implement our band detector, which used CRP in the positive regulation route and the TetR inverter (part BBa_Q04400) and cI (part BBa_C0051) in the negative one (see Fig. 1).

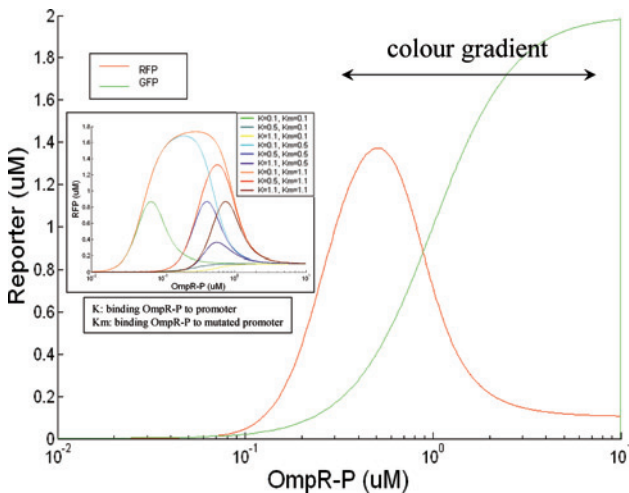


Fig. 6 Simulated dynamics of the two reporters of our system (RFP-red line, and GFP-green line) versus the OmpR-P concentration, which is the input of the actuator device

In the inset, we show a sensitivity analysis of the RFP versus the binding affinity with the ompC promoters.

This results in an RFP expression band at a given OmpR-P concentration. In addition, GFP expression is under direct control of OmpR-P by using a YES gate (see Fig. 1). In this way, when the two output concentrations are added, we obtain a graded response as a function of the different vanillin concentration levels. To perform our simulations (see Fig. 6), we used the following protein concentration effective model

$$\begin{aligned}
 \frac{d[TetR]}{dt} &= \alpha_{TetR} \frac{([OmpR - P]/K_{OmpR-P/PompCm})^{n_{OmpR-P/PompCm}}}{1 + ([OmpR - P]/K_{OmpR-P/PompCm})^{n_{OmpR-P/PompCm}}} \\
 &\quad - \beta_{TetR}[TetR] + \gamma_{TetR} \\
 \frac{d[cI]}{dt} &= \alpha_{cI} \frac{1}{1 + ([TetR]/K_{TetR/Ptet})^{n_{TetR/Ptet}}} \\
 &\quad - \beta_{cI}[cI] + \gamma_{cI} \\
 \frac{d[CRP]}{dt} &= \alpha_{CRP} \frac{([OmpR - P]/K_{OmpR-P/PompC})^{n_{OmpR-P/PompC}}}{1 + ([OmpR - P]/K_{OmpR-P/PompC})^{n_{OmpR-P/PompC}}} \\
 &\quad - \beta_{CRP}[CRP] + \gamma_{CRP} \\
 \frac{d[RFP]}{dt} &= \alpha_{RFP} \frac{([cI]/K_{cI/Pand})^{n_{cI/Pand}}}{1 + ([cI]/K_{cI/Pand})^{n_{cI/Pand}}} \\
 &\quad \times \frac{([CRP]/K_{CRP/Pand})^{n_{CRP/Pand}}}{1 + ([CRP]/K_{CRP/Pand})^{n_{CRP/Pand}}} \\
 &\quad - \beta_{RFP}[RFP] + \gamma_{RFP} \\
 \frac{d[GFP]}{dt} &= \alpha_{GFP} \frac{([OmpR - P]/K_{OmpR-P/PompCm})^{n_{OmpR-P/PompCm}}}{1 + ([OmpR - P]/K_{OmpR-P/PompCm})^{n_{OmpR-P/PompCm}}} \\
 &\quad - \beta_{GFP}[GFP] + \gamma_{GFP}
 \end{aligned}$$

where α_i is the rate synthesis of gene i , β_i its

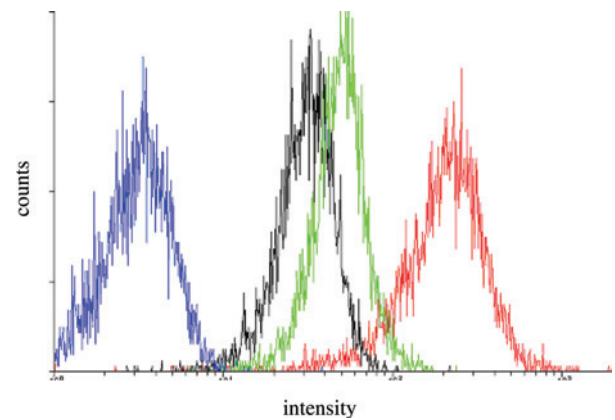


Fig. 7 FACS histogram to characterise ompC promoters (natural and mutated) by using a GFP as reporter

Blue line = negative control, a culture of XL1-Blue
 Black line = construction using the mutated ompC promoter
 Green line = construction using natural ompC promoter
 Red line = green fluorophore, which is a positive control



Fig. 8 UPV-UV Valencia iGEM 2006 team members: A. Jaramillo, G. Rodrigo, J. Urchueguia, C. Mata, C. Aroca, J. Carrera, D. Giménez, C. Edo, A. Montagud, E. Navarro, J.V. Medrano, M. Baguena, A. Ferrando, G. Fuertes, C. Navarrete, A. Aparici, P. Fernandez de-Cordoba (up-down, left-right)

degradation rate, and γ_i its basal rate. K_{ij} is the regulation coefficient from gene j to i , and n_{ij} is the Hill coefficient.

3 Materials and methods

Plasmids were derived from pUC containing antibiotic resistance. The CDS of the vanillin receptor was obtained using DNA synthesis. For the synthesis of the AND promoter [5], we used DNA synthesis from oligos [9]. The CDS for the CRP and Trg were obtained from genomic PCR. The growth medium was LB at 37°C with the corresponding antibiotic (ampicillin or kanamycin). The fluorescence of our reporters has been measured by using FACS (see Fig. 7). We constructed the hybrid protein Trz by digestion at the NdeI restriction site [4].

For the vanillin receptor, we applied an automated protein design method [3] that uses the high-resolution atomic structure of a protein in order to find all possible sequences stabilising the corresponding fold and protein-ligand complex. We used an x-ray structure (pdb code 2DRI) for the closed D-ribose binding protein (rbsB). We screened mutations around the active site while preserving residues needed for the allosteric motion as in [2]. We then simulated our actuator device and performed a parametrical sensitivity analysis (see Fig. 6) by using Matlab and Simulink [10]. We have implemented our constructions using parts from the Registry [6] and standard assemblies [7].

4 Conclusions

Our team (Fig. 8) has designed, simulated and partially constructed a modular genetic system that is expected to give a graded response according to vanillin concentration. For this purpose, we have obtained a cellular biosensor by using a phosphorylation mechanism to connect the membrane receptor with

the genetic network. As compared to the circuit used by Weiss' group on their work on pattern generation [11], the use of a two-regulator promoter allows for signal integration and reduces the number of genes required for our device. These types of promoter will be very useful for future synthetic circuits, due to their small sequence size but sophisticated behaviour and their integration ability. Our computational methodology is general and can be used to sense alternative small molecules. Finally, the standardised sensor-mediator part and the circuit design can be modified or generalised for future biosensor projects.

5 Acknowledgments

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The 'Cell-See-Us' cellular thermometer

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Abstract: We report the design, construction, and characterisation of a five-component cellular fluorescence-based thermometer and its individual parts, built from the bottom up in *Escherichia coli*. The sensing mechanism is based on the temperature-sensitive regulatory activity of the mutated LacI_{ts} transcription factor. Such microscale temperature sensors have potential applications in the measurement of temperature gradients on 2D and 3D contours and in microelectromechanical system (MEMS) devices.

1 Goal

In recent years, the engineering of biological systems has taken on a bottom-up, black-box approach that is analogous to the hierarchical method of building complex computer systems from simple, functionally-defined components [1–3]. The goal of the Cell-See-Us project is to follow this systematic approach to produce a proof-of-principle *E. coli* thermometer. Smaller and simpler genetic components are characterised and interconnected to form a larger system with the properties of a thermometer. Fluorescent reporter proteins are used to produce a visible response to the environmental temperature. Microscale, fluorescence-based thermometers are advantageous where contact thermometers are impractical or where measurements from a large number of spatially distributed sites is desirable. For example, potential applications can be found in microscale total analysis systems [4], MEMS, and in studying heat distributions.

2 Design

The Cell-See-Us thermometer is composed of 20 basic genetic parts from the BioBrick registry. Conceptually, the system can be viewed as consisting of five components: an arabinose-controlled sensitivity adjuster, a temperature-sensitive LacI_{ts}-based inverter, a tetR-based inverter, and green fluorescent protein (GFP) and monomeric red fluorescent protein (mRFP) reporters. The sensitivity adjuster is connected to LacI_{ts} inverter, whose output is connected to mRFP and tetR inverter; the tetR inverter output is connected to GFP (Fig. 1).

The thermometer construct is switched on by the addition of L-arabinose to the cell culture. L-arabinose enters cells and binds to an endogenous *E. coli* protein, AraC, that normally represses the pBad promoter [5]. Once the pBad repression is relieved, transcription of the LacI_{ts} gene may then proceed. The intracellular level of LacI_{ts} protein determines the activity level of the thermometer. Therefore, by controlling pBad promoter activity using L-arabinose, the Cell-See-Us thermometer sensitivity can be adjusted.

The LacI_{ts} inverter is at the heart of the Cell-See-Us thermometer. The inverter consists of the LacI_{ts} coding region and a hybrid pLac promoter. LacI_{ts} protein is produced under pBad activation and forms dimers and tetramers that exist in equilibrium with each other [6]. The tetramers are the active form. When they bind to the pLac promoter, they inhibit transcription. Modifying the temperature causes a shift in the dimer-tetramer equilibrium and, in turn, the activity of the pLac promoter. This temperature sensitivity is the basis of the cellular thermometer.

At low temperatures, there is a high ratio of LacI_{ts} tetramers to dimers. The tetramers bind to and repress the pLac promoter, thereby preventing the transcription of the tetR gene. In turn, the lack of TetR protein production allows the pTet promoter immediately downstream to upregulate the expression of GFP. Simultaneously, the second pLac in the construct is also repressed, decreasing the intracellular levels of mRFP. The higher amount of GFP produced relative to mRFP results in net green fluorescence.

Conversely, when cells are exposed to high temperatures, LacI_{ts} predominantly exists in the dimer form. With a lower intracellular concentration of tetramers, repression of the two pLac promoters is relieved. The first pLac causes tetR to be expressed. The TetR protein then binds to and represses the pTet promoter controlling GFP expression. The second pLac promotes the transcription and expression of mRFP. This results in net red fluorescence.

Hence, low temperatures make the cells fluoresce green, and high temperatures make the cells fluoresce red. Temperatures in between result in a combination of green and red, which appears orange or yellow (Fig. 1). In addition to basic temperature sensitivity, the system also features three external control points for tuning and testing. First, the level of LacI_{ts} production can be controlled through the level of L-arabinose. Second, excess

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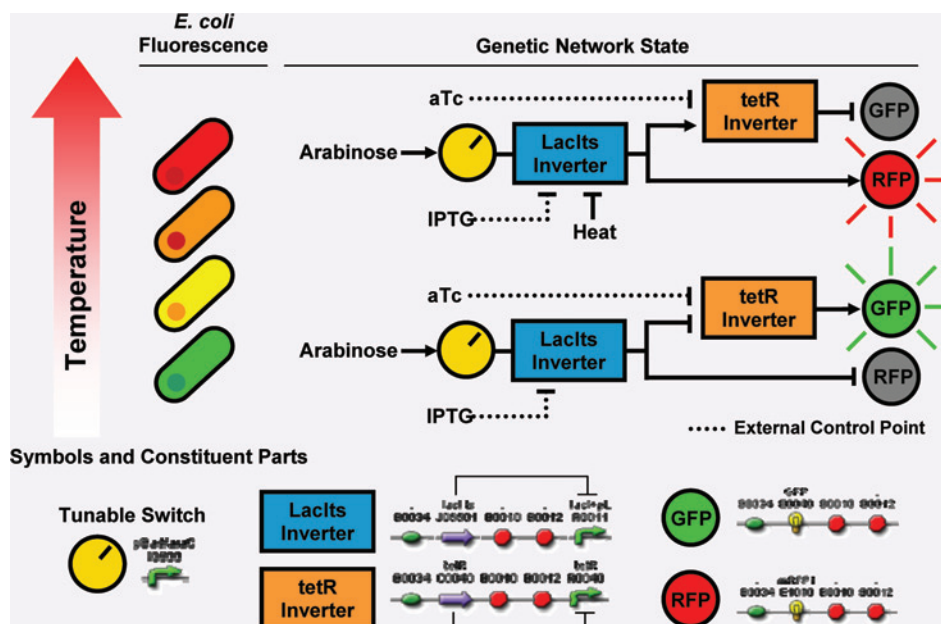


Fig. 1 Systems diagram

Cell-See-Us thermometer changes color as the LacIs Inverter responds to temperature change

LacIs can be inactivated by the addition of IPTG, which binds to LacIs dimers and inhibits tetramerisation. Finally, analogous to the IPTG inhibition of LacIs, aTc can be used to control the intracellular levels of TetR

protein. These control points allow us to compensate for system variations caused by factors such as promoter leakiness and ribosome binding site strength.

The modular approach makes our design flexible and easy to test. For example, removal of the tetR inverter and GFP results in a one-colour thermometer that measures temperature by mRFP fluorescence intensity. Individual parts can also be investigated by test constructs.

3 Parts characterisation

3.1 Method

E. coli transformed with the test vector were grown for at least 16 h at 37°C in LB broth (Bioshop, Burlington, Ontario). The grown cell culture was diluted to an optical density (OD₆₀₀) of 0.1 (FLUOstar OPTIMA) and treated with the appropriate chemical inducer. Each sample was incubated at 37°C in a shaker for 4-8 h until 0.8 OD₆₀₀ was reached. The fluorescence was then measured (at 520 nm with 485 nm excitation for GFP; at 612 nm with 584 nm excitation for mRFP) and normalised against OD₆₀₀.

3.2 Characterised constructs

Two constructs, BBa_J5517/J5518 and BBa_J5524 (Fig. 5), were used to characterise three functional components: the pBad switch, the LacIs inverter, and the tetR inverter.

3.3 Results

To test the functionality of the pBad promoter as a tunable switch, cells transformed with construct BBa_J5517 were treated with varying levels of L-arabinose, and the fluorescence was measured. Inducing the pBad promoter is expected to increase the production of LacIs, which represses GFP production. The decrease in normalised fluorescence intensity with increasing levels of L-arabinose (Fig. 2a) followed the predicted behaviour (Fig. 2e), providing evidence for the functionality of the pBad switch, the LacIs inverter, and the GFP reporter.

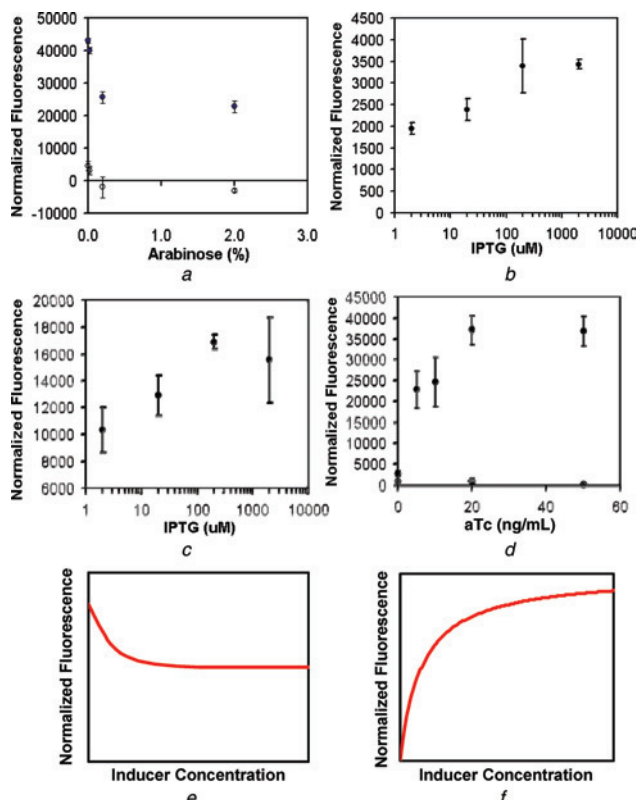


Fig. 2 Parts characterisation

a Reduction in fluorescence due to arabinose-induced LacIs increase and GFP repression in part BBa_J5517 (●) and DH5α control (○)

b IPTG inhibition of LacIs repression on GFP in BBa_J5517

c mRFP in BBa_J5518

d Fluorescence increase induced by aTc inhibition of tetR at 0.2% arabinose in part BBa_J5524 (●) and DH5α control (○)

e Predicted trend for Fig. 2a based on mathematical modelling

f Predicted trend for Fig. 2b based on modelling

$n = 4$ for all data points

Error bars represent \pm one standard error

Further characterisation of the LacIts inverter was performed using the external IPTG control point. IPTG prevents tetramerisation of LacIts by binding to LacIts dimers. The observed fluorescence increase with increasing IPTG provides additional confirmation for the production of LacIts protein (Fig. 2b–c).

Finally, functionality of the tetR inverter was investigated through the external aTc control point, using construct BBa_J5524. Cells were treated with 0.2% L-arabinose to induce transcription of tetR. Similar to the IPTG-LacIts system, aTc is expected to bind to TetR and reduce pTet repression. GFP could then be produced. Experimentally, increasing the level of aTc was shown to increase the level of fluorescence. This indicates that TetR is present in the system and is inhibiting the production of GFP (Fig. 2d).

This series of tests indicate the correct general input-output trend for each component. By building the more complex construct BBa_J5524 based on the simpler construct BBa_J5517/J5518, we were able to isolate and test the tetR inverter functionality. This systematic approach is instrumental in successfully building functional devices.

4 Mathematical modelling

A deterministic mathematical model was constructed to predict the behaviour of the Cell-See-Us thermometer. All interactions of the system are broken down into a set of chemical kinetic equations, including promoter binding, transcription, translation, protein-protein binding, and degradation. The Law of Mass Action was used to describe the rate of change of each of the intracellular species with respect to time. Solving this system of equations using estimated parameters produces rough predictions in the general trend of the system and sub-system behaviours (Figs. 2e–f, 3b, 4b).

5 Thermometer device characterisation

5.1 Method

E. coli transformed with the testing vector were grown for at least 16 h at 37°C in minimal media. The grown cell culture was diluted to an OD₆₀₀ of 0.1. After 37°C incubation for 2–3 h, the cell culture was removed to its testing

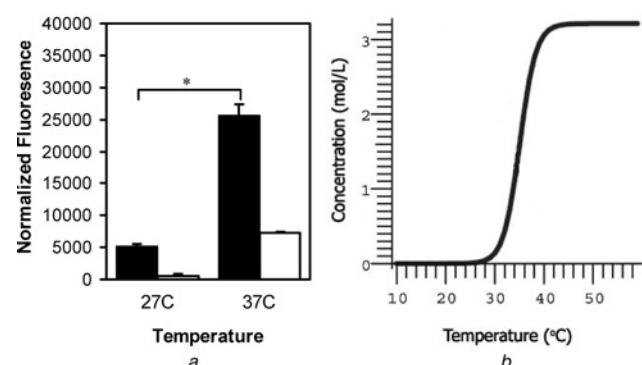


Fig. 3 Single-color thermometer characterisation

a mRFP fluorescence intensity change when part BBa_J5518 (■) and DH5α control (□) were cultured at 27°C and 37°C, respectively. Test conducted in 0.2% arabinose. n = 4. Error bars represent ± SEM. (*P < 0.01)
b Model-predicted trend of GFP intracellular concentration increase with increasing temperature for BBa_J5518

temperature and grown until an OD₆₀₀ of 0.8 was reached for fluorescence measurement.

5.2 Characterised devices

The single-colour thermometer BBa_J5518 and the dual-colour Cell-See-Us BBa_J5530 were characterised for temperature sensitivity.

5.3 Results

In addition to being a test construct, BBa_J5518 also functions as a single-colour thermometer device. The device was investigated for sensitivity between 27°C and 37°C. Instead of IPTG induction, temperature was used to alter the level of available LacIts tetramer repressors to achieve fluorescence regulation. The results show a highly significant fluorescence intensity increase (p < 0.01) with temperature, agreeing with predictions (Fig. 3).

To characterise the Cell-See-Us dual-colour thermometer, cells were removed to a plate with a concentric temperature gradient after 2 h incubation at 37°C and allowed to grow for 18 h. The temperature gradient was created by resting the plate on a coin heated to 40°C. The colour of cell fluorescence was observed to change from red to green radially outward, according to the gradient (Fig. 4a). Finally, image analysis was performed to map the contours of red-to-green intensity ratio on the plate (Figure 4c). The results indicate a functional dual-colour cellular thermometer that fluoresces red when hot, green when cold, and shades of orange in between.

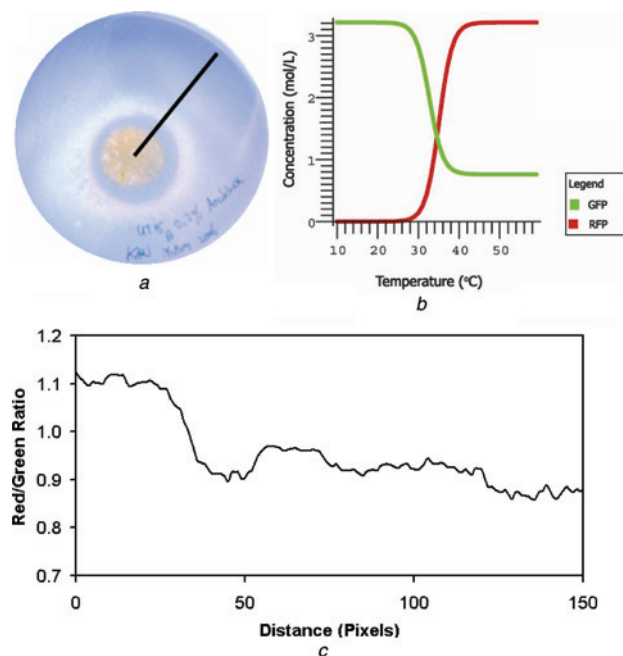


Fig. 4 Cell-See-Us thermometer characterisation

a Cells cultured on a plate with heat gradient created by resting the plate on a coin heated at 40°C. Image shows red contrast between center and rim
b Predicted trends for GFP and mRFP behaviour with increasing temperature based on modelling
c Ratio between red and green values along black line in A. Image analysis done using ImageJ software (National Institute of Mental Health, Bethesda, MD)

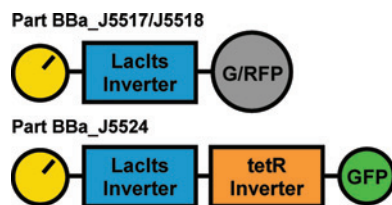


Fig. 5 The characterised constructs

6 Conclusions

Through a bottom-up, black-box approach, we have successfully constructed both a single-colour and a dual-colour cellular thermometer in *E. coli*. The Cell-See-Us thermometer has demonstrated continuous temperature



Fig. 6 The Blue Water 2006 iGEM team

sensitivity as measured by fluorescence colour change. The project served as a demonstration of the powerful approach of building a complex system from characterised, simple genetic components. The iGEM competition was a wonderful experience for our team (Fig. 6).

7 Acknowledgments

The authors would like to thank the Blue Water 2005 team, in particular A. Hessel, E. Nazereth, J. Yang and M. Scoot for their pioneering efforts on the cellular thermometer project. The team acknowledges The University of Toronto Engineering Society, The Edward S. Rogers sr. Department of Electrical and Computer Engineering, University of Toronto Students' Union, University of Waterloo Mathematics Endowment Fund (MEF), University of Waterloo Department of Biology, and Waterloo Engineering Endowment Fund (WEEF) for funding.

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Hydrogen detector testing and design using synthetic biology and fluorescence photo analysis

B.P. Flynn, M.-H. Ho, R. Morris, S.D. To, D.-P. Ma and T. French

Abstract: Synthetic biology methods can be used to construct a biological hydrogen detector, provided a hydrogen activated promoter can be identified. The *hybB* operon is directly involved in the production of hydrogenase II and thus the *hybB* promoter may show some activity based on hydrogen presence. This report studies the relationship between *hybB* promoter activity and hydrogen concentration as well as the possible testing methods for a biological hydrogen detector. The *hybB* promoter can be placed upstream of DNA coding for a part that converts PoPS (polymerases per second) into fluorescence to create a detector with reporting ability. This engineered DNA, when inserted into an effective host, in this case *E. coli*, can be tested and characterised based on fluorescence. The *hybB* promoter, like most promoters, is not a '0–1' promoter that only has a discrete 'on' or 'off' state. The range of transcription rates possible will allow quantification over a range of hydrogen concentrations. The product was tested using a digital camera and pixel analysis. This method, once perfected, will provide a method for measuring fluorescence in altered environments or in remote locations without access to a spectrophotometer.

1 Background

Mississippi State University participated in the iGEM competition in 2006 for the first time. It is worth noting that the constructive atmosphere of the iGEM Jamboree, the newness of the field, and the cooperative nature of the different teams as they shared and learned from each other where participation was more important than 'competition'. While the iGEM Jamboree does involve a competition with multiple categories, it is more closely akin to a research symposium. MSU's involvement in synthetic biology started when Dr S.D. Filip To, Undergraduate Coordinator for the Department of Agricultural and Biological Engineering at Mississippi State University, took interest after speaking with iGEM founder Dr Drew Endy at an IBE conference in Atlanta in 2005. Dr To added the bio-energy research effort at MSU to his list of research projects in 2006. On attending another IBE convention and hearing about the progress of synthetic biology, he saw a connection between the emerging field of synthetic biology and his bio-energy research. With visions of the discoveries to come using synthetic biology to improve upon alternative energy resources, he jumped

into the new field feet first and carried his department with him. 'As I learned about the discipline I began to understand the magnitude of the challenges in synthetic biology. But what is life without challenge?' he explained. Dr. To worked to enlist professors, graduate students and undergraduate students from several departments to field MSU's first iGEM team and start a project. The possibilities as well as the exhilaration with which Dr. To explained the new field left most students and faculty with a desire to learn more.

2 Aims of the project

Since this was MSU's first year in the competition, and we did not have a base of existing research in synthetic biology, our goals were to learn the procedures for synthetic biology and to establish an experienced team through an introductory project. Year-end goals were to build a network between several departments, create an interest club and begin work towards a synthetic biology seminar or special topic class.

All of these goals, and our ability to move forward in the new field, relied on our ability to choose a project and produce results. This success would provide both a starting platform for future research and an experienced team capable of working towards more advanced projects.

Our project was to test the hydrogen-dependent promoter activity of the *hybB* operon by designing, building and testing a cellular scale biological hydrogen detector. We chose a hydrogen detector because our department is already strongly rooted in bio-energy, and we have long term goals of designing biological machines to be used in the production and refinement of different fuels. While current mechanical hydrogen detectors are available, a biological hydrogen detector could be implemented directly in the same cells used for hydrogen production, thus providing visible quantification.

We tested our constructions along the way by checking the plasmid and segment lengths using standard isolation and gel electrophoresis techniques. However, we also

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needed to develop testing procedures to test the hybB composite detector in altered gaseous environments. Standard testing procedures for fluorescence call for the sample to be placed in a cuvette or on a micro plate and analysed in a spectrophotometer. Our goal in testing was to develop a novel testing method that could be performed quickly without disturbing the detector.

3 Description of the work

We chose to work with the hybB promoter because the hybB operon is directly involved in the production of hydrogenase II, which catalyses the oxidation of hydrogen. A biological feedback mechanism should exist to relate hybB promoter activity to hydrogen presence. Though we were uncertain of the exact relationship between hydrogen presence and hybB promoter activity, we hoped to show that the promoter could be used outside of the context of the hybB operon as part of a hydrogen-controlled biological machine.

Our choice to work with the hybB promoter expedited our experiment because we had the part sequence and primers readily available from the UCSF iGEM team. UCSF's work on a biological thermometer provided supporting information on using the hybB promoter in synthetic biology, and allowed us to work quickly towards something novel as we developed our team and synthetic biology program.

We constructed our primary machine from three registry parts using standard synthetic biology assembly procedures. Routine methods were used for cell inoculation, growth and subculture, transformation, isolation, gel electrophoresis, enzymatic digestion and ligation.

We used the hybB promoter (part Bba_J45503), which was used by the UCSF iGEM team in 2005 as a cold-shock promoter, a lacI quad-part inverter (part Bba_Q04121) and a PoPS-to-YFP converter (Bba_E0430).

The hybB promoter was not available from the MIT Registry of Parts, so we constructed the promoter using the sequence offered to us by UCSF. The hybB promoter was cloned from *E. coli* using colony PCR with primers provided to us by UCSF. The product was sequenced to confirm successful amplification of the hybB promoter. The hybB promoter's cold shock function has been characterised as most active at 30°C and inactive at 37°C.

The quad-part-inverter (QPI) was added to the design to provide greater sensitivity control. The lacI QPI contains a strong ribosome binding site, a protein coding region for lacI, a two-part transcription terminator and the PLlac promoter, in that order. A QPI is an inverter because the downstream promoter is inhibited by the product of the coding region. In our case, the protein coding region codes for lacI which inhibits the PLlac promoter. Thus an 'on' signal passed into the quad part inverter will be inverted to an 'off' signal.

The YFP converter contains a strong ribosome binding site, coding sequence for yellow fluorescence protein and a stop site, in that order. This part converts PoPS, or polymerases per second, to fluorescence. YFP was reported to have absorbance and emission peaks around 515 nm and 530 nm, respectively, so we used green light to excite the YFP.

The complete part (Fig. 1) was designed as an inverted fluorescence hydrogen detector. The PLlac promoter in the lacI QPI is normally active and with the addition of IPTG could be induced to higher levels, up to approximately 600-fold (iGEM registry Bba_R0011 and DH5-alpha-Z1). In the presence of hydrogen the hybB promoter would become active and lacI would be produced,

inhibiting the PLlac promoter and fluorescence. Changes in IPTG concentration should allow control over the sensitivity of the sensor.

We also designed an analogous part using the tetR QPI (part Bba_Q04400) and the same hybB promoter and YFP reporter. Unlike the lacI QPI, the tetR QPI has no inducer. The part design would naturally fluoresce and would inactivate itself in the presence of hydrogen, like the lacI part, but could not be induced to a more active 'on' state to compensate for the inhibiting factor tetR produced when the hybB promoter was active due to hydrogen concentration or temperature. This part did not make it very far past construction because we re-examined the designs after construction and decided the part would not provide us with as broad a range of operation, and thus would not provide conclusive data as to whether or not the hybB promoter was a hydrogen activated promoter.

All antibiotic concentrations were 50 µg/ml. Part E0430, the PoPS-to-YFP converter, was obtained in PSB1A2, so ampicillin was used when working with this part and plasmid. The tetR and lacI QPIs, Q04400 and Q04121, are found in pSB1K3, which contains resistance to kanamycin, so kanamycin was used when working with this plasmid. pSB1K3 is 4425 base pairs long without any extra parts. The large size results in a reduced yield of the plasmid. Though construction is technically possible in any order, construction efficiency can be improved slightly if it is possible to eliminate pSB1K3 from the process in early steps.

4 Testing

Standard methods of isolation and gel electrophoresis were used to verify construction steps by size. Quantification of hydrogen concentrations using our system requires characterisation of the relationship between hydrogen concentration, temperature and fluorescence. Measurement of fluorescence requires quantification of the wavelength and intensity of absorption and emission. The usual device used for these measurements is a spectrophotometer. A spectrophotometer can emit and detect across a broad range of wavelengths and chart the absorption and emission peaks of a sample. Most spectrophotometers generate results for a sample placed in a cuvette or on a microplate.

Testing our part proved more complicated than the normal procedures because testing required controlled atmospheric conditions. This immediately ruled out plates for the testing procedures. Cuvettes were also ruled out because we did not immediately have the ability to control the atmosphere inside the cuvettes.

Another issue of concern during testing was the hybB promoter's classification as a cold shock promoter by the UCSF iGEM team. They used the promoter to develop a thermometer using different fluorescent proteins to indicate different temperature, and according to their data, the promoter is most active at 30°C and inactive at 37°C. With our experimental design and testing procedures, this characteristic should not have had an affect on our data if we maintained a steady temperature during each data collection step.

While trying to design testing procedures, we contacted several departments on campus looking for environmentally sealed test booths to fill with differing hydrogen concentrations. Large containers with mixtures of hydrogen and oxygen can be very dangerous. This danger was overcome when we were introduced to volatile organic analysis (VOA) vials by Dr. Todd French in the Chemical Engineering Department. We were then able to work with relatively small amounts of the hydrogen-air mixtures.

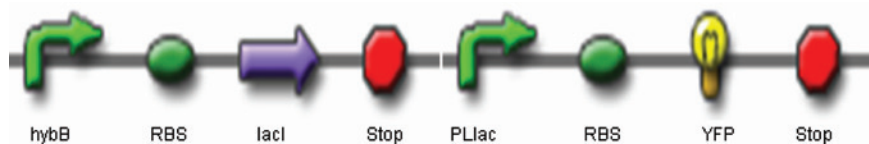


Fig. 1 Part BBa_J43001 components

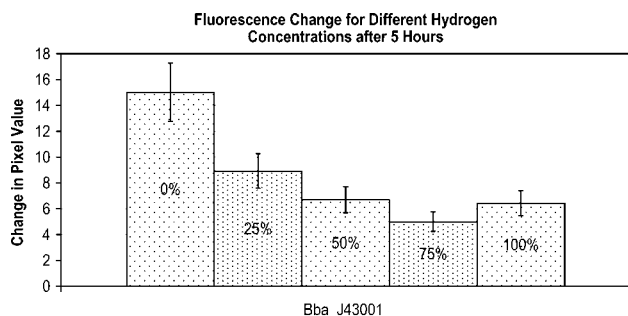


Fig. 2 Fluorescence change for different hydrogen concentrations after 5 h

Bacterial cells were streaked on a horizontal slants in VOA vials to maximise the surface area. These vials have a volume of 40 ml and an airtight septum top which will allow for exchange of contents using a syringe many times without losing the airtight quality. Once growth was close to uniform, the vials were adjusted to differing hydrogen concentrations using a vacuum pump, a syringe and bottled hydrogen to achieve final concentrations of 0%, 25%, 50%, 75% and 100% hydrogen and the remaining volumes regular air.

In a dark container we exposed the products to light near the absorbance wavelength of the reporter, 515 nm, and used a digital camera with fixed settings to photograph the fluorescence. Pictures were taken immediately before, immediately after, 5 h after and 24 h after hydrogen introduction.

Fluorescence was measured using pixel analysis in MATLAB. Pixel values were averaged over a selected area containing dense *E. coli* growth. Comparing visual

assessment of fluorescence with MATLAB analysis, we determined a standard scale and direction for fluorescence analysis. Data for sample fluorescence was compared to control data for wild-type *E. coli*. For several experimental runs pictures were taken with both wild-type *E. coli* and the engineered machine samples in the same pictures for truer comparisons. For each sample the 5 h pixel value was normalised to the initial value to find the change in fluorescence. Then sample fluorescence changes were normalised to wild-type fluorescence changes. The results shown in Fig. 2 reveal varying fluorescence changes for different hydrogen concentrations. The experiment and data collections were performed at a near constant room temperature, so these normalisation procedures minimise temperature induced changes in promoter activity.

5 Conclusions

The MSU iGEM (Fig. 3) team successfully constructed and tested a synthetic biological machine. A full work plan is presented in Fig. 4. Several departments combined efforts, equipment and ideas to work towards a research effort in synthetic biology that continues to grow at MSU.

Initial testing of the hydrogen detector showed differences in fluorescence between the constructed machine and wild-type *E. coli*. These differences were visible to the unaided eye and quantifiable using pixel analysis. Analysis also showed fluorescence change varied for different hydrogen concentrations. The procedures for testing and analysis still need to be refined to accurately quantify the results and characterise the promoter activity.



Fig. 3 The Mississippi State 2006 iGEM team

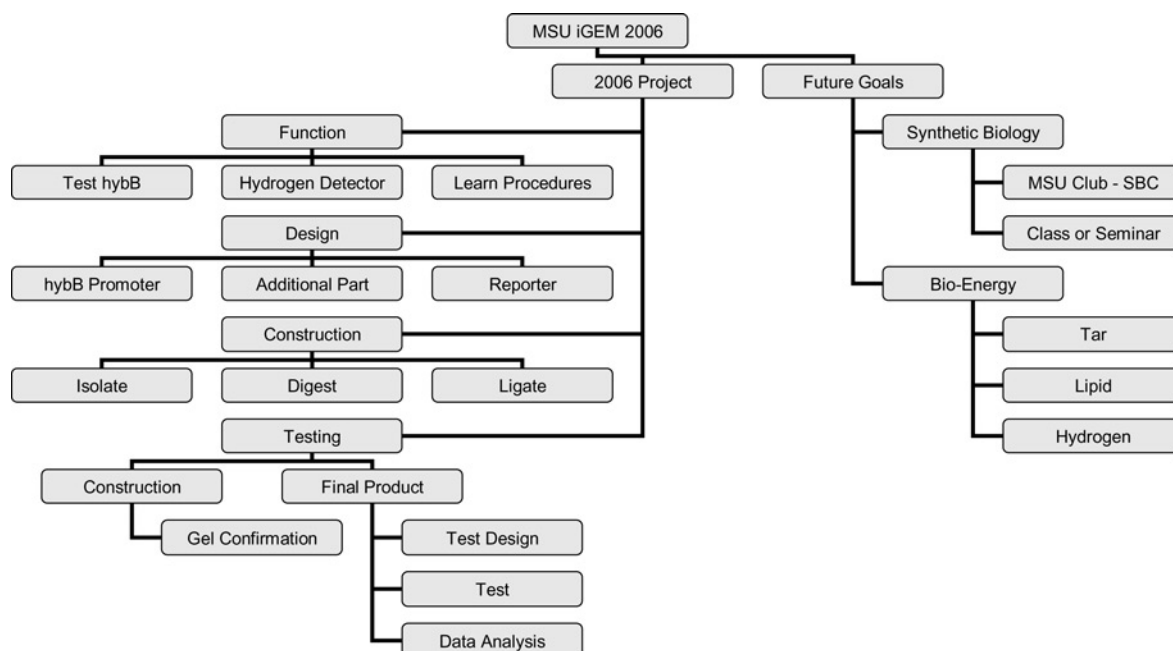


Fig. 4 MSU iGEM 2006 work chart

6 Mississippi State 2006 iGEM team

6.1 Faculty

- Dr S. D. Filip To, Agricultural and Biological Engineering
- Dr Din-Pow Ma, Biochemistry
- Dr Todd French, Chemical Engineering
- Dr Bob Reese, Electrical and Computer Engineering

6.2 Students

- Meng-Hsuan Ho graduate student, Molecular Biology
- Brendan Flynn graduate student, Biomedical Engineering

- Robert Morris graduate student, Biological Engineering
- Teri Vaughn Undergraduate, senior, Biomedical Engineering
- Courtney Harbin Undergraduate, senior, Biochemistry
- Lauren Beatty Undergraduate, junior, Biomedical Engineering
- Paul Kimbrough Undergraduate, junior, Biological Engineering
- Joseph Chen Undergraduate, junior, Biological Engineering
- Scott Tran Undergraduate, junior, Biological Engineering
- Sam Pote Undergraduate, freshman, Biological Engineering

Development of a novel biosensor for the detection of arsenic in drinking water

J. Aleksic, F. Bizzari, Y. Cai, B. Davidson, K. de Mora, S. Ivakhno, S.L. Seshasayee, J. Nicholson, J. Wilson, A. Elfick, C. French, L. Kozma-Bognar, H. Ma and A. Millar

Abstract: We sought to develop a whole-cell biosensor for the detection of arsenic in drinking water, a major problem in Bangladesh and West Bengal. In contrast to previously described systems, our biosensor would give a pH change as output, allowing simple detection with a pH electrode or pH indicator solution. We designed and modelled a system based on the arsenate-responsive promoter of the *Escherichia coli* arsenic detoxification system, using urease to increase pH in the absence of arsenate, and β -galactosidase (LacZ) to decrease pH in the presence of arsenate. The pH-reducing β -galactosidase part of the system was constructed and tested, and was found to give a clear response to arsenate concentrations as low as 5 ppb arsenic, well below the World Health Organisation (WHO) recommended limit of 10 ppb.

1 Initial project ideas

In our initial discussions, we considered a variety of projects, including a biosensor for detection of water contamination, and a hybrid biological-electrical device such as a variable resistor. Ultimately we decided to combine these two ideas to develop a whole cell biosensor that responds to arsenic by producing a measurable pH change which can be easily detected with a pH electrode.

2 Usefulness of a biosensor for arsenic in drinking water

Arsenic contamination in drinking water is a serious problem in many parts of the world, and is particularly associated with Bangladesh and West Bengal, where many tube wells were inadvertently drilled through arsenic bearing sediments, resulting in drinking water contaminated with arsenate (AsO_4^{3-}) and arsenite (AsO_3^{3-}) anions [1–3]. Consumption of water with elevated arsenic levels over a prolonged period leads to arsenicosis, resulting in skin lesions and various cancers. Many millions of people worldwide are at risk. The current WHO recommended limit for drinking water is 10 ppb arsenic; in many countries a more relaxed limit of 50 ppb is still in operation. Current field tests for arsenic detection are not altogether satisfactory, requiring toxic chemicals and having a significant false negative rate.

A simple, cheap and sensitive field assay for arsenic levels would therefore be extremely useful. A whole cell microbial biosensor, with an arsenic-responsive promoter

linked to a suitable reporter gene, might be one way of achieving this [4, 5]. Arsenic biosensors have been previously reported [for example, 6, 7], but have mainly relied on luminescent or fluorescent reporter genes, which require expensive equipment and trained technicians, and are not really suitable for field use. Other biosensors have used the LacZ/Xgal reporter system, but this is difficult to quantify, and Xgal (5-bromo-4-chloro-3-indoyl- β -D-galactoside) is expensive and requires refrigeration. By contrast, a sensor giving a pH response would allow a simple quantitative measurement using a cheap pH electrode or solid state device (ISFET, ion-sensitive field effect transistor), or even just a pH indicator solution giving a colour change.

3 Design of the system

We devised a system based on the plasmid-encoded arsenic resistance operon of *Escherichia coli*. This is controlled by two repressor proteins, ArsR (responding to low concentrations of arsenate or arsenite) and ArsD (responding to higher concentrations)[8, 9]. Each is negatively autoregulated. To induce an increase in pH, we chose to use urease, which breaks down urea, $(\text{NH}_2)_2\text{CO}$, to release ammonium ions. This is used in diagnostic microbiology to distinguish urease-positive bacteria such as *Proteus*, since the pH can rise above 9. To induce a decrease in pH, we chose to use *lacZ*. This encodes β -galactosidase, which catalyses the essential first step in the fermentation of lactose to acetic and lactic acids (mixed acid fermentation) in *E. coli* and related organisms. This reaction is also used in diagnostic microbiology, since the pH can fall below 4.5.

In our design, the activity of the biosensor is initiated by exposure to lactose. Urease is expressed from a hybrid promoter repressed by both lambda cI repressor and LacI repressor. In the presence of lactose, but absence of arsenate, urease is induced and the pH rises. When low amounts of arsenate are present, an ArsR-repressed promoter is induced, leading to expression of lambda cI repressor, switching off urease production. Thus the pH remains neutral. If higher amounts of arsenate are present, *lacZ* expression is induced through an ArsD-responsive

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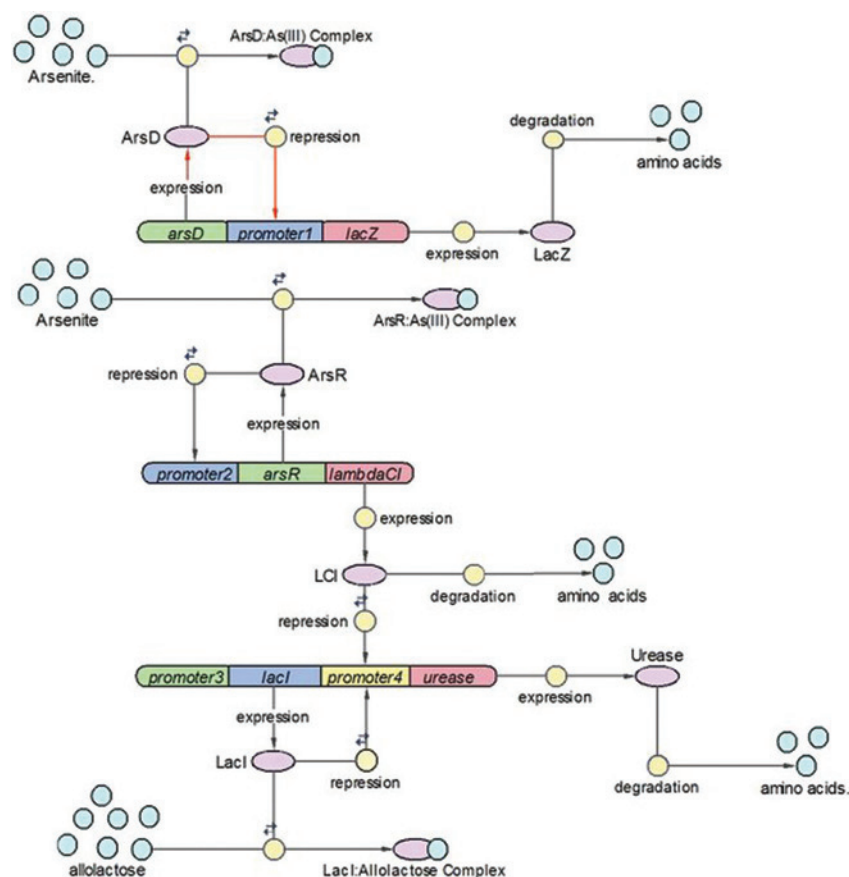


Fig. 1 Schematic diagram of the complete biosensor system

promoter, leading to a fall in pH. By using multiple promoters in this way, a high sensitivity and high dynamic range (the range of arsenate concentrations over which arsenate concentration can be estimated from the response) are achieved. A schematic diagram of the system is shown in Fig. 1.

4 Modelling

This system was modelled using an ordinary differential equation (ODE)-based model, with parameters estimated based on the literature. The model showed good induction of urease and repression of *lacZ* in the absence of arsenate, and repression of urease and induction of *lacZ* at high arsenate levels. Sensitivity analysis was also conducted in order to determine which parameters had the greatest effect on the urease and *lacZ* responses. For example, it was found that the parameter having the greatest effect on the steady-state level of *lacZ* expression in the presence of arsenate was the degradation rate of ArsD.

5 Testing the concept

To demonstrate that a detectable pH change could be achieved in the laboratory, we constructed a BioBrick bearing the *E. coli* chromosomal *ars* promoter and negatively autoregulated *arsR* gene (BBa_J33201). The chromosomal *ars* operon is similar to the plasmid-encoded one we had originally envisaged using, but is controlled solely by ArsR and has no equivalent of the second repressor, ArsD [10, 11]. We also made a BioBrick (BBa_J33202) of the *lacZ'* gene encoding the N-terminus of *lacZ*, which complements the *lacZ*ΔM15 mutation found on the chromosome of laboratory strains of *E. coli* such as JM109 and XL1Blue. These BioBricks were joined to generate

BBa_J33203. Unfortunately, we were not able to obtain template DNA for the plasmid encoded *arsR* and *arsD* genes we had intended to use within the time frame of the competition. We therefore also cloned the *ars* promoter and *arsR* gene from *Bacillus subtilis* [12], to test whether this might have a sufficiently different affinity for arsenate to be useful in this context. This was joined to *lacZ'* to generate BBa_J33206. In experiments using JM109/pSB1A2-BBa_J33203, concentrations of arsenate as low as 5 ppb gave a significant decrease in pH at incubation times above 5 h, persisting to over 20 h, in a non-optimised medium based on Luria-Bertani medium with 2% w/v lactose (Fig. 2). The response was easily detected with a pH electrode and could also be visually assessed using the pH indicator methyl red, which has a pKa around 4.8–5.0. The equivalent system using BBa_J33206 unfortunately did not show a response to arsenate, with even arsenate-free controls giving a rapid drop in pH, suggesting

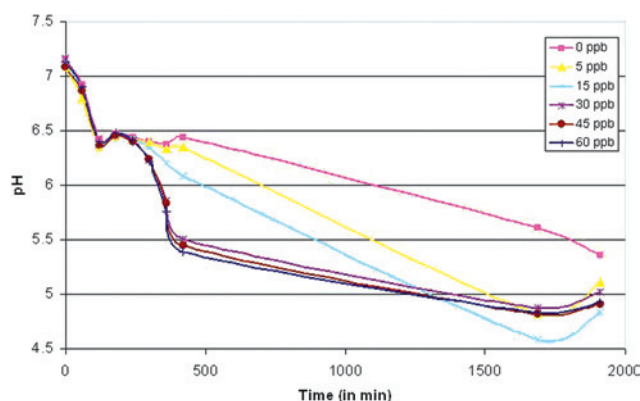


Fig. 2 pH response of *E. coli* JM109/pSB1A2-BBa_J33203 to varying arsenate concentrations

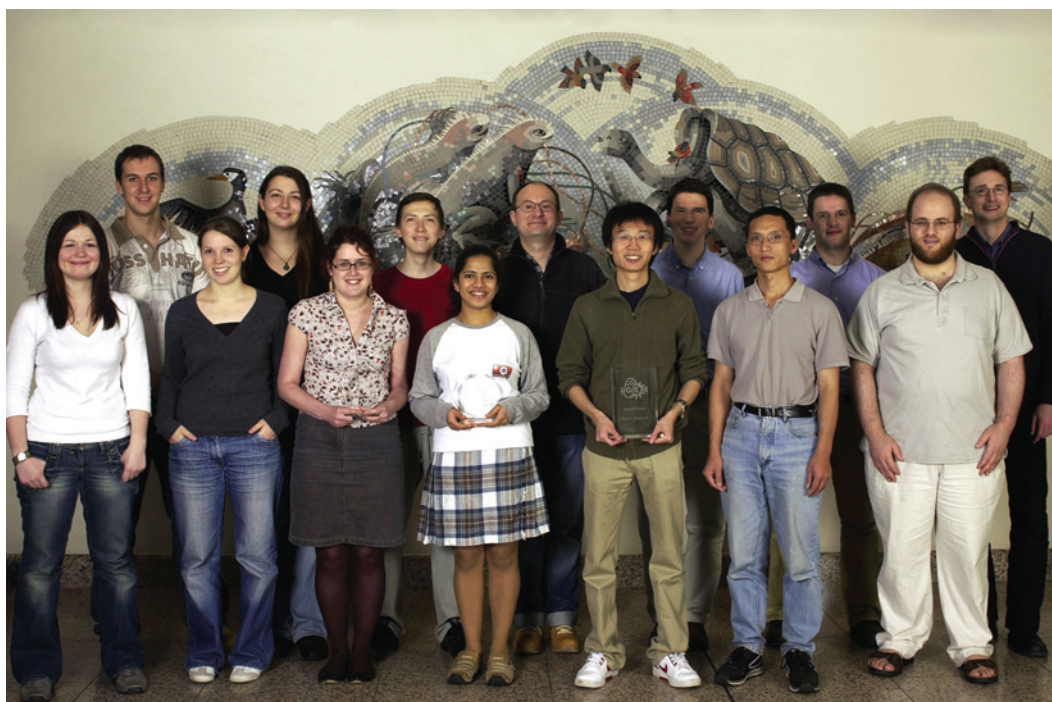


Fig. 3 The University of Edinburgh iGEM2006 team from left to right: Jennifer Wilson; Kim de Mora; Bryony Davidson; Jelena Aleksic; Judith Nicholson; Sergii Ivakhno; Sreemati Lalgudi Seshasayee; Dr Laszlo Kozma-Bognar; Yizhi Cai; Dr Chris French; Dr Hongwu Ma; Dr Alistair Elfick; Farid Bizzari; Prof. Andrew Millar

high background activity due to incomplete repression of this promoter in *E. coli*.

6 Generation of other parts

For our system, we also needed a urease part to increase pH in the absence of arsenate. The most obvious choice would have been the urease gene cluster present in some strains of *E. coli*; however, this consists of around 5 kb of DNA with seven genes (*ureDABCEFG*, where *ureABC* are the genes encoding the urease subunits, and the other genes encode accessory factors required for proper insertion of the nickel cofactor) and contains six forbidden restriction sites which would have to be mutated out individually before the gene cluster could be converted to a BioBrick. After searching the literature, we found that the *Bacillus subtilis* urease gene cluster consists of only three genes, *ureABC*, which can nevertheless be assembled into a functioning urease in *E. coli* without the requirement for the usual accessory proteins [13]. Unfortunately, this gene cluster also contains two forbidden restriction sites, *EcoRI* and *SpeI*.

To check that this urease would be suitable for our purposes, the *ureABC* region was cloned in pGemT-easy (Promega) and pBluescript SK+ (Stratagene). In both constructs, activity was demonstrated in *E. coli*, with pH rising to 9 after incubation in LB with 0.2% w/v urea. Having decided that this urease would be suitable, we used site-directed mutagenesis to remove the two forbidden restriction sites. This was successfully achieved, but the mutant gene cluster gave no detectable urease activity. Sequencing revealed a possible frameshift mutation in *ureC* as well as two non-silent single nucleotide changes as compared to the published sequence. Thus we were unable to generate a urease BioBrick during the time available.

The final part required for our system was the hybrid promoter repressed by both lambda cI and LacI. This was generated by fusing the P_{RM-P_R} region of bacteriophage



Fig. 4 The University of Edinburgh iGEM 2006 team logo

lambda, including cI binding sites OR1, OR2 and OR3, to the 3' end of the lac promoter region including the LacI binding site. The N-terminal region of *lacZ* was also included, so that *lacZ*' expression could be used to regulate the promoter. This BioBrick was designated BBa_J33205. Unfortunately, we did not have time to build the constructs necessary to test the regulation of this part.

7 Conclusions

Even though we were not able to build our complete design in the time available, we have demonstrated that a simpler version, *E. coli* JM109/pSB1A2-BBa_J33203, gives a good pH response to arsenate concentrations as low as 5 ppb arsenic, with a dynamic range in the region of 0–20 ppb, in a non-optimised system. This can be detected with a pH electrode or a pH indicator (methyl red) which

changes from yellow to red when the pH falls below about 5. Recalling that the WHO limit is 10 ppb, this device is suitable for further development, and could potentially be the basis for a cheap and useful sensor to help prevent the ongoing tragedy of chronic arsenic poisoning. Also, we have submitted the functioning arsenic-responsive promoter to the Registry (BBa_J33201), so we hope that others may be inspired to develop even better arsenic biosensors in the future.

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